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16185

Cultivation of *Toxoplasma* in the Developing Chick Embryo.*

JOHN O. MACFARLANE AND ISAAC RUCHMAN. (Introduced by Albert B. Sabin.)

From the Department of Bacteriology, University of Cincinnati, College of Medicine.

Reports of the cultivation of toxoplasma in the developing chick embryo have appeared in the literature but these accounts are brief and give little experimental data. Levaditi, *et al.*,¹ and Wolfe, Cowen, and Paige² stated that inoculated fertile eggs hatched but that the chicks died within several days and their tissues were parasitized. Weinman³ found

that numerous small white nodules developed on the chorio-allantoic membrane after chorio-allantoic or yolk sac inoculation. In the above investigations recently isolated strains of toxoplasma were used for egg inoculation; deaths appeared to have been infrequent for they were not mentioned specifically. The data to be presented demonstrate that a strain of human origin, well adapted to mice, not only readily infected eggs but also produced such uniform embryo deaths as to make the embryonated egg a satisfactory titration medium.

The "R.H." strain of toxoplasma isolated by Sabin⁴ from a fatal human case of encephalitis was used in these studies. It was

* This study was supported by a grant-in-aid from the United States Public Health Service.

¹ Levaditi, C., Sanchez-Bayarri, V., Lepine, P., and Schoen, R., *Ann. de l'Inst. Pasteur*, 1929, **43**, 673.

² Wolfe, A., Cowen, D., and Paige, B. H., *J. Exp. Med.*, 1940, **71**, 187.

³ Weinman, D., *Puerto Rico J. Pub. Health and Trop. Med.*, 1944, **20**, 125.

⁴ Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

maintained in this laboratory by continuous intracerebral passages in mice and the 304th to 344th passage material was used for initiating the cultures in embryonated eggs. Fertile hen's eggs from pullorum-tested flocks were inoculated into the yolk sac. Incubation of the eggs both before and after inoculation was at 37°C. LD₅₀ titers in mice and chick embryos were calculated according to the method of Reed and Muench.⁵

Injection of toxoplasma-infected, mouse brain suspensions into the yolk sac of embryonated eggs on at least 6 different occasions led to multiplication of the parasites with resulting death of the embryo in 4 to 10 days after inoculation. Examination of the dead embryos revealed the presence of numerous yellow-white plaques, 0.5 to 3 mm in diameter, on the chorio-allantoic and amniotic membranes (Fig. 1). The areas surrounding the lesions were thickened and histologically represented regions of dense cellular infiltration containing numerous toxoplasma (Fig. 2). Smears of the chorio-allantoic membrane and the yolk sac stained with Wright's stain revealed numerous toxoplasma both free and intracellular.

Three series of egg passages were studied in order to seek evidence of adaptation or change.



FIG. 1.

Toxoplasma in the developing chick embryo. Eggs 18 days old.
Left. Infected chorio-allantoic membrane.
Right. Normal chorio-allantoic membrane.

One series was carried through 27 consecutive passages in 10- or 11-day-old fertile eggs (Table I). A second series was carried through 5 passages in 9- or 10-day incubated eggs, and three egg passages were done in which 6- or 7-day incubated eggs were utilized. No evidence was obtained that continuous or multiple egg passages altered the length of time between inoculation and death of the embryo. Most of the deaths occurred between the 5th and 6th days. Titrations performed in mice showed that LD₅₀ titers for the various egg passages were about the same. Titrations of the various egg tissues revealed that while the chorio-allantoic membranes contained the greatest number of parasites the yolk sacs had only slightly less. Organisms were present in small number in the embryonic fluids (LD₅₀ titers of 10^{-2.0} to 10^{-3.0}) and the viscera of embryos (stained smears). Membranes harvested either from dead or living chick embryos at the time of the maximum number of embryo deaths possessed high titers (LD₅₀ titers of 10^{-4.5} to 10^{-5.0}) while the membranes of living eggs harvested two days before this maximum time gave lower titers (10^{-3.5}).

Although toxoplasma multiplied well in fertile hen's eggs between 6 and 11 days of age deaths in the younger embryos were spread over a larger number of days, *i.e.*, 4 to 8 days.

Several comparative titrations were performed in mice and chick embryos and the results are listed in Table II. In 3 of the tests higher LD₅₀ titers were obtained in chick embryos. In these cases the amount injected into eggs was proportionately larger. However when similar inocula were used, *i.e.*, 0.5 ml per egg via the yolk sac and 0.5 ml per mouse intraperitoneally, the LD₅₀ titers were identical. When the actual number of LD₅₀ doses per gram of tissue were calculated the values obtained in three of the tests were slightly higher in mice than in chick embryos.

Other studies revealed that storage of infected membranes at 4°C yielded viable organisms for periods up to one month. Attempts to cultivate toxoplasma in the pooled embryonic fluids from 17-day fertile eggs were

⁵ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

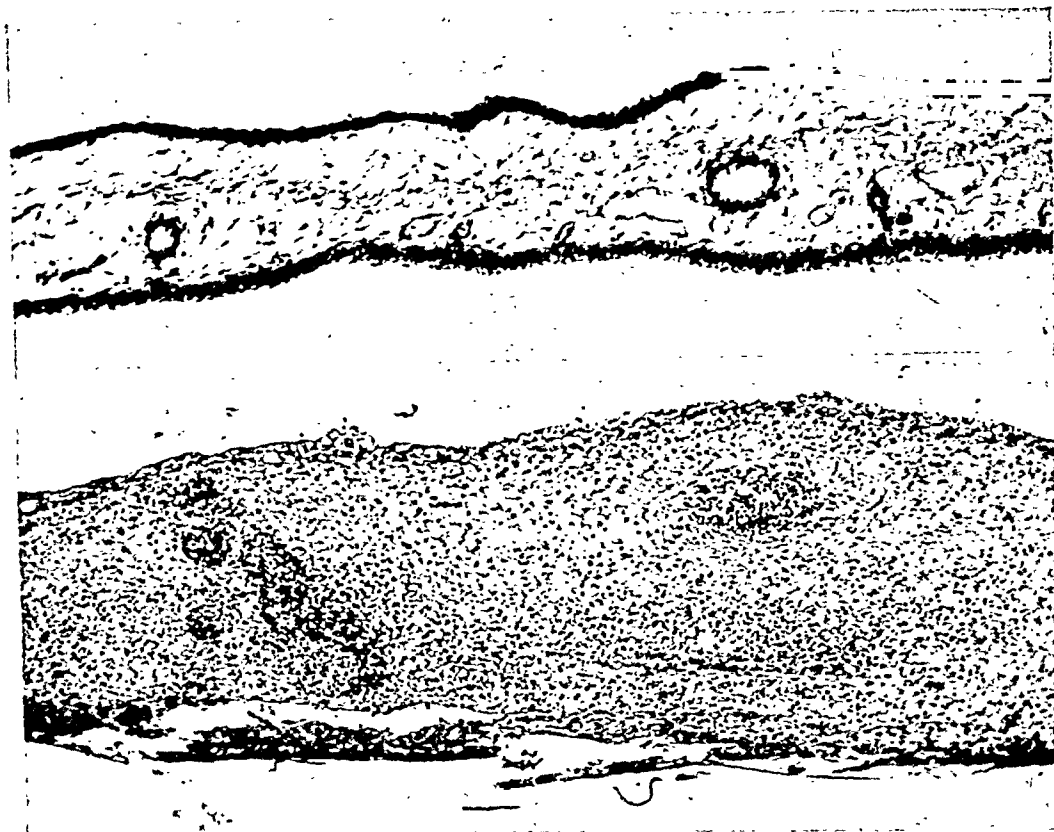


FIG. 2.
Toxoplasma in the developing chick embryo. Eggs 18 days old. H. & E. $\times 160$.
Upper. Normal chorio-allantoic membrane.
Lower. Infected chorio-allantoic membrane.

TABLE I.
Toxoplasma Passages in Eggs Inoculated via the Yolk Sac.
0.5 ml per egg in 10- and 11-day-old embryonated hen's eggs.

Egg passage	Tissue suspension employed	Day of death	Intracerebral LD ₅₀ titers in mice		
			Yolk sac	Chorio-allantoic membrane	Membrane pool*
1	10% mouse brain	5,5,6,6,6,6,7,7,7	10-3.5	10-4.0	
4	5% Y.S.-C.A.*	6,6,6,6,6			10-4.5
7	10% " "	5,6,6,6			10-5.5
10	" " "	4,5,6,6,6,6,6,6			10-5.0
12	" " "	4,4,5,5,5,5,5			10-4.5
15†	" " "	6,6,6,10			10-4.0
20	" " "	5,5,5,5,6,6,6,6			10-5.0
27	" " "	6,6,6,6,6	10-3.5	10-4.5	

* Pool of yolk sac and chorio-allantoic membrane.

† Inoculated with membranes which had been stored 5 days at 4°C.

unsuccessful. More than 13 passages through eggs did not result in any obvious modification of pathogenicity for animals. Inocula-

tion of infected embryonal tissues caused disease in mice, rats, chicks, rabbits, and a rhesus monkey. Preliminary results indicated

TABLE II.

Comparative Titrations of Toxoplasma Infected Tissues in Mice and Developing Chick Embryos.
10-day chick embryos inoculated via the yolk sac. Mice inoculated intracerebrally.

Passage	Inoculation			LD ₅₀ titer	LD ₅₀ doses per g of indicated tissue, × 1000
	Tissue	Amt, ml	Titration in:		
335	Mouse brain	.5 .03	Embryos Mice	10-5.0+ 10-4.5	200+ 1,000
336	" "	.5 .03	Embryos Mice	10-5.0 10-4.0	200 330
344	" "	.5 .5*	Embryos Mice	10-4.5 10-4.5	64 64
15	Y.S.-C.A.	.5 .03	Embryos Mice	10-4.0 10-3.0	20 33

* Mice in this case injected intraperitoneally.

that neutralization tests could be performed in the chick embryo.

The embryonated hen's egg may be an adjunct for the primary isolation of toxoplasma and although inoculation of eggs with the laboratory-adapted strain gave uniformly good results the probability is that for the initial recovery of strains the mouse is still the animal of choice.

Summary. A laboratory-adapted strain of

toxoplasma was successfully propagated in the embryonated hen's egg. Uniform mortality was obtained and the majority of deaths occurred between 5 and 6 days after inoculation. The concentration of organisms in the chick embryo gave LD₅₀ titers of 10^{-4.5} to 10^{-5.0} which approximated that usually attained in mice. Repeated passage through eggs resulted in no modification of pathogenicity for animals.

16186 P

Histochemical Differentiation Between Esterases.*

GEORGE GOMORI.

From the Department of Medicine, The University of Chicago.

Strikingly different properties of esterases (lipases) obtained from various species^{1,2} have been reported by a number of workers. The differences include substrate specificity,³ stereochemical specificity,⁴ behavior towards activators and inhibitors, and pH optima.⁵

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

¹ Rona, P., and Bach, E., *Bioch. Z.*, 1920, **11**, 166.

² Gyotoku, K., *Bioch. Z.*, 1928, 1939.

³ Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, **50**, 183.

⁴ Rona, P., and Ammon, R., *Bioch. Z.*, 1927, **181**, 49.

Owing to multiple overlapping of differences and similarities between enzymes obtained from various sources, it is still undecided whether the various effects are due to the existence of several well defined individual enzymes or to the presence of unidentified accompanying substances.

With a histochemical method for the visualization of sites of lipase activity, similar differences between the esterases of various organs were found.

Experimental. Organs of 8 freshly killed white mice (5 males and 3 females) were fixed in chilled acetone, dehydrated, embedded

⁵ Davidsohn, H., *Bioch. Z.*, 1913, **49**, 249.

and stained according to the technique previously published.^{6,7} In addition to the original substrates, a new one, G-9096-CJ (Atlas Powder Co., Wilmington, Del.), a stearic ester of modified mannitan, was used. This new substrate seems to be hydrolyzed more rapidly than the related Tweens, but the localization and the relative intensity of the reaction in various organs is the same with all substrates. Pieces of practically all organs (liver, kidney, spleen, lung, heart, stomach, intestine, pancreas, salivary glands, testis, uterus, ovary, urinary bladder, etc.) were included in a single paraffin block, and serial sections were incubated with the substrate to which the substances to be tested were added.

The following substances were selected for their known effects on esterases: cholate,^{8,9} quinine,¹⁰⁻¹³ arsanilate,^{1,10,11,13} eserine,^{4,14} pilocarpine,⁴ urethane,¹⁵ caprylate,¹⁶ n-butyraldehyde,¹⁷ acetophenone,¹⁷ hexylresorcinol¹⁸ and NaCl.¹⁹

Results. Since a strict quantitative evaluation of histochemical reactions of this type is impossible, increase or decrease in the in-

⁶ Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 362.

⁷ Gomori, G., *Arch. Path.*, 1946, **41**, 121.

⁸ Willstätter, R., and Memmen, F., *Z. physiol. Chem.*, 1924, **133**, 229.

⁹ Willstätter, R., and Memmen, F., *Z. physiol. Chem.*, 1924, **133**, 247.

¹⁰ Rona, P., and Pavlovic, R., *Bioch. Z.*, 1922, **130**, 225.

¹¹ Rona, P., and Pavlovic, R., *Bioch. Z.*, 1923, **134**, 108.

¹² Rona, P., and Takata, M., *Bioch. Z.*, 1923, **134**, 118.

¹³ Rona, P., and Haas, H. E., *Bioch. Z.*, 1923, **141**, 222.

¹⁴ Mendel, B., and Rudney, H., *Bioch. J.*, 1943, **37**, 59.

¹⁵ Rona, P., and Lasnitzki, A., *Bioch. Z.*, 1925, **163**, 197.

¹⁶ Weber, H. H. R., and King, C. G. J., *J. Biol. Chem.*, 1935, **108**, 131.

¹⁷ Weinstein, S. S., and Wynne, A. M., *J. Biol. Chem.*, 1935-36, **112**, 649.

¹⁸ Glick, D., and King, C. G., *J. Biol. Chem.*, 1932, **97**, 675.

¹⁹ Glick, D., *Nature*, 1941, **148**, 662.

TABLE I.
Effect of Various Substances on Lipase (Esterase) Activity.

	Liver	Pancreas	Kidney	Intestine	Stomach	Testis		
						Interstitial cells	Spermatic elements	Lung
Quinine, .005 to .01 M	1—	2— to 3—	1— to 2—	3—	2— to 3—	0 to 1—	0 to 1—	0 to 3—
Arsanilate, .0002 M	3—	1—	3—	3—	1—	3—	0 to 1—	2— to 3—
Taurocholate, .02 M	2— to 3—	2+ to 3+	3—	3—	2+ to 3+	2—	2—	2— to 3—
Pilocarpine, .01 M	0	0	0 to 1—	0 to 1—	0 to 1—	0 to 1—	0 to 1—	0 to 1—
Urethane, 1 M	0	0	0	0	0	0	0	0
Eserine, .002 to .005 M	0 to 1—	0 to 1—	0 to 2—	0 to 2—	0 to 2—	0	0	0
Caprylate, .005 M	2— to 3—	1—	3—	2—	1—	2—	2—	2—
n-Butyraldehyde, .005 M	0	0	0	0	0	0	0	0
Acetophenone, .005 M	0	0	0	0	0	0	0	0
Hexylresorcinol, .0005 M	0	0	0	0	0	0	0	0
NaCl, 1.2 M	0	0	0	0	0	0	0	0

The symbol 3— stands for complete suppression of enzyme activity.

tensity of the reaction will be indicated only by plus and minus signs, with the arbitrary degrees 1, 2 and 3. (Table I). The pattern of response was markedly uniform in all animals, with the exception of the lung which behaved erratically towards quinine (no inhibition, one case; slight to moderate inhibition, 3 cases; complete suppression of the reaction, 4 cases).

The histochemical results are in good agreement with previous findings on the *in vitro* behavior of hepatic and pancreatic lipase towards quinine and arsanilate and on the similarity between the pancreatic and gastric enzymes, both being activated by bile salts.⁹ On the other hand, the effect of several substances, markedly active in test tube experiments, such as urethane, butyraldehyde, acetophenone, hexylresorcinol and NaCl, cannot be observed under the conditions of the histochemical experiment. An interesting new

finding is the difference in sensitivity to arsanilate between the enzyme of the interstitial cells (testis) and that of the spermatogenic elements. Another curious observation was a marked diffusion of the reaction around the sites of activity in the pancreas and the stomach, but nowhere else, when cholate was added to the substrate. In some cases such large numbers of lead sulfide granules were embedded in the protecting collodion membrane and on its surface as to make the exact localization of the enzyme virtually impossible. This finding may indicate an increased diffusibility of pancreatic and gastric lipase in the presence of bile salts.

Summary. Differences in the behavior of esterases (lipases) from various sources towards activator and inhibitor substances, similar to those found previously in *in vitro* experiments, can be observed also in tissue sections stained for lipase.

16187

Biological Activity of Crystalline Procaine Penicillin *In vitro* and *In vivo*.*

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In a recent communication Salivar, Hedger, and Brown¹ described the preparation and chemical properties of crystalline procaine penicillin. The present report deals with the biological activity and with the absorption and excretion of this form of penicillin and of other relatively insoluble salts of penicillin.

Materials and Methods. Unless otherwise

specified, crystalline procaine penicillin G and dihydro-F, prepared from crystalline sodium penicillins G and dihydro-F, respectively, were used throughout this study. The potencies of these preparations were determined by the Oxford cup plate method;² the per cent G, non-G penicillin by a modification of the N-ethyl piperidine method.³ Sensitivity determinations were carried out by a quantitative broth dilution technic. The method of Tompsett, Schultz, and McDermott,⁴ using *Strepto-*

* The authors wish to express their appreciation to Mr. C. J. Salivar, Mr. O. Sumner, Mr. W. Armstrong, and Dr. P. Regna for preparation of the samples used in this study. They are indebted also to Mr. F. H. Hedger for chemical analyses of the preparations and to Mrs. W. Reed and Mrs. D. Rinne for assistance in carrying out the many biological assays necessary for this study.

¹ Salivar, C. J., Hedger, F. H., and Brown, E., *J. A. C. S.*, 1948, in press.

² Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

³ *Federal Register*, April 4, 1947, v. **12**(67), p. 2222, 141.5 (f).

⁴ Tompsett, R., Schultz, S., and McDermott, W., *J. Bact.*, 1947, **53**, 581.

coccus hemolyticus as the test organism and crystalline sodium penicillin G as standard, was used for determination of the concentrations of penicillin in blood and urine. Mouse protection tests were carried out using *Streptococcus hemolyticus* and *Diplococcus pneumoniae* as the test organisms and the method described by Hobby *et al.*⁵ in a recent communication.

Activity of Crystalline Procaine Penicillin In Vitro. Two preparations of procaine penicillin G, having potencies of 1066 and 1025 units per mg, and 2 preparations of procaine penicillin dihydro-F, having potencies of 1010 and 987 units per mg, were used.[†] Likewise 3 preparations containing mixtures of crystalline procaine penicillins G and dihydro-F as well as certain naturally occurring penicillin pigments were used.[‡] These showed potencies of 930 u/mg, 975 u/mg, and 950 u/mg, respectively. The sensitivity of a variety of organisms to these penicillins was determined as follows:

Six-hour plain broth cultures of *Streptococcus hemolyticus* (strain C230Mv), *D. pneumoniae* (strain I/230), *Staphylococcus aureus* (strain H), *Bc. subtilis*, *Streptococcus viridans*, *E. coli*, and *A. aerogenes* were used throughout. Cultures were diluted with broth to a constant density immediately prior to use. A density equivalent to a MacFarland BaSO₄ No. 1 standard and allowing 78% transmission on a Photovolt Lumetron No. 400 was arbitrarily chosen as standard. For each gram negative organism tested a series

of 9 tubes were set up containing 0.1, 0.15, 0.2, 0.25 . . . 0.5 ml of broth containing 400 units of procaine penicillin per ml. In a few instances, a concentration of 800 units per ml was essential. For the more sensitive gram positive organisms, concentrations of 0.1 unit per ml were used. The total volume of each tube was adjusted to 0.5 ml with sterile broth and 0.5 ml of a 10⁻³ dilution of the standardized culture was then added to each. The final concentration of organisms was, therefore, about 150,000 per ml. Incubation was carried out at 37°C for a period of 24 hours. The sensitivity of an organism was accepted as the least amount of penicillin causing complete inhibition of growth, as evidenced by absence of gross turbidity, after 24 hours incubation.

Crystalline sodium penicillins X, G, dihydro-F, and K[§] were tested simultaneously for their activity against the same group of organisms.

As shown in Table I, crystalline procaine penicillins G and dihydro-F are highly effective antibacterial agents *in vitro*. The activity of crystalline procaine penicillin G at times may differ quantitatively, however, from that of the crystalline sodium salt of penicillin G. Under the experimental conditions used in this study, all organisms tested were slightly more sensitive to crystalline sodium penicillin G than to crystalline procaine penicillin G. No differences were observed in the sensitivities of these organisms to the sodium salt of penicillin dihydro-F as

⁵ Hobby, G. L., Burkhart, B., and Hyman, B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 296.

[†] The theoretical potencies of crystalline procaine penicillins G and dihydro-F are calculated to be 1041 and 986 units per mg respectively, based on crystalline sodium penicillins G and dihydro-F at 1667 and 1600 units per mg, respectively. Each mg of crystalline procaine penicillins G and dihydro-F contains 0.42 and 0.43 mg procaine base, respectively.

[‡] On crystallization of procaine penicillins G and dihydro-F from impure penicillin containing approximately 70 to 80% G and 20 to 30% dihydro-F, certain of the naturally occurring impurities are precipitated with the penicillins. Nevertheless the penicillins appear to be in the crystalline form.

[§] The crystalline sodium salts of penicillin used were identical with the preparations used by one of the present investigators (G.L.H.) in a previous study and have been described in detail elsewhere.⁵ The preparation of crystalline penicillin G had a potency of 1634 units per mg by the bioassay method and a *Bc. subtilis*, *Staphylococcus aureus* differential ratio of 1.0. The polariscopic assay of this preparation was 1635 units per mg. Ultra-violet absorption indicates 100% G. The crystalline penicillin K used showed a potency of 2182 units per mg and a differential ratio of 0.36; the crystalline penicillin X, a potency of 1069 units per mg, and a differential ratio of 1.39; the purified penicillin dihydro-F, a potency of 1675 units per mg and a differential ratio of 0.57.

TABLE I.
Comparative Activity of Crystalline Procaine and Sodium Penicillins *in Vitro*.

Organism	Sensitivity in units per ml						
	Procaine penicillins			Sodium penicillins			
	G	Dihydro-F	Mixed*	G	Dihydro-F	X	K
<i>D. pneumoniae</i> (I/230)	0.085	0.042	0.033	0.030	0.040	0.005	0.022
<i>Strep. hemolyticus</i> (C203Mv)	0.022	0.020	0.015	0.015	0.025	0.004	0.012
<i>Strep. viridans</i>	0.220	0.200	0.150	0.100	0.200	0.075	0.100
<i>Staph. aureus</i> (H)	0.100	0.050	0.047	0.050	0.050	0.060	0.040
<i>Bc. subtilis</i>	0.025	0.035	0.018	<0.005	0.035	0.005	0.025
<i>A. aerogenes</i>	110.000	120.000	53.000	40.000	160.000	80.000	>200.000
<i>E. coli</i>	160.000	160.000	87.000	60.000	200.000	40.000	>200.000

* Crystalline procaine penicillins G and dihydro-F mixed, with accompanying naturally-occurring impurities.

compared to its procaine salt. Furthermore, their sensitivities to crystalline sodium penicillin G and to mixtures of crystalline procaine penicillins G and dihydro-F, with accompanying impurities, were identical.

Procaine penicillin may be prepared by the interaction of procaine hydrochloride and sodium penicillin. Procaine hydrochloride in low concentration has in itself no bacteriostatic action against this group of test organisms. It is chemically derived from para-aminobenzoic acid, and has been shown by Woods and Fildes⁶ and by others,⁷⁻¹¹ to be capable of inhibiting the *in vitro* and *in vivo* bacteriostatic action of sulfadiazine against *Streptococcus hemolyticus* and certain other organisms. The possibility that procaine at times may serve as an essential metabolite and thus alter the concentration of penicillin necessary for inhibition of growth was, therefore, suggested. Procaine hydrochloride, however, in concentrations of 0.02, 1.0, and 100 mcg per ml failed to alter the sensitivity of *Streptococcus hemolyticus* to crystalline sodium penicillins G or dihydro-F.

Activity of Crystalline Procaine Penicillin

In Vivo. Preparations of crystalline procaine penicillins G and dihydro-F in oil, diluted to contain 1,000 units of penicillin per ml, were used throughout this study. In a few instances preparations containing mixtures of crystalline procaine penicillins G and dihydro-F, with certain of the accompanying impurities of partially purified penicillin, were used. Crystalline potassium penicillin G in oil and beeswax was also tested for comparison.

Fifteen-hour blood broth cultures of a highly virulent strain of Group A hemolytic streptococcus (strain C203Mv) and of pneumococcus type I (Strain I/230) were used throughout. Mice were infected by the intraperitoneal route with one cc of 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} dilutions of culture. Treatment was carried out by the subcutaneous route, a single injection of penicillin being administered 2 hours after infection. All experiments were controlled with a series of untreated animals. In all instances the penicillins used were administered in sesame or peanut oil, a concentration of 1,000 units of penicillin per ml being used.

As shown in Table II, procaine penicillin is an effective chemotherapeutic agent against hemolytic streptococcal and pneumococcal infections. Three hundred units of procaine penicillin G, administered in a single injection 2 hours after infection, is adequate to protect approximately 70% of animals against 10 to 10,000 lethal doses of hemolytic streptococci or pneumococci. Similar protection against hemolytic streptococcal infections was ob-

⁶ Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

⁷ Woods, D. D., and Fildes, P., *Chem. and Industry*, 1940, **50**, 133.

⁸ Boroff, D. A., Cooper, A., and Bullock, J. G. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 182.

⁹ Casten, D., Fried, J. J., and Hallman, F. A., *Surg. Gynecol. Obstet.*, 1943, **76**, 726.

¹⁰ Keltch, A. K., Baker, L. A., Kralh, M. E., and Clowes, G. H. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 533.

¹¹ Legge, J. W., and Durie, E. B., *Med. J. Australia*, 1943, **20**, 561.

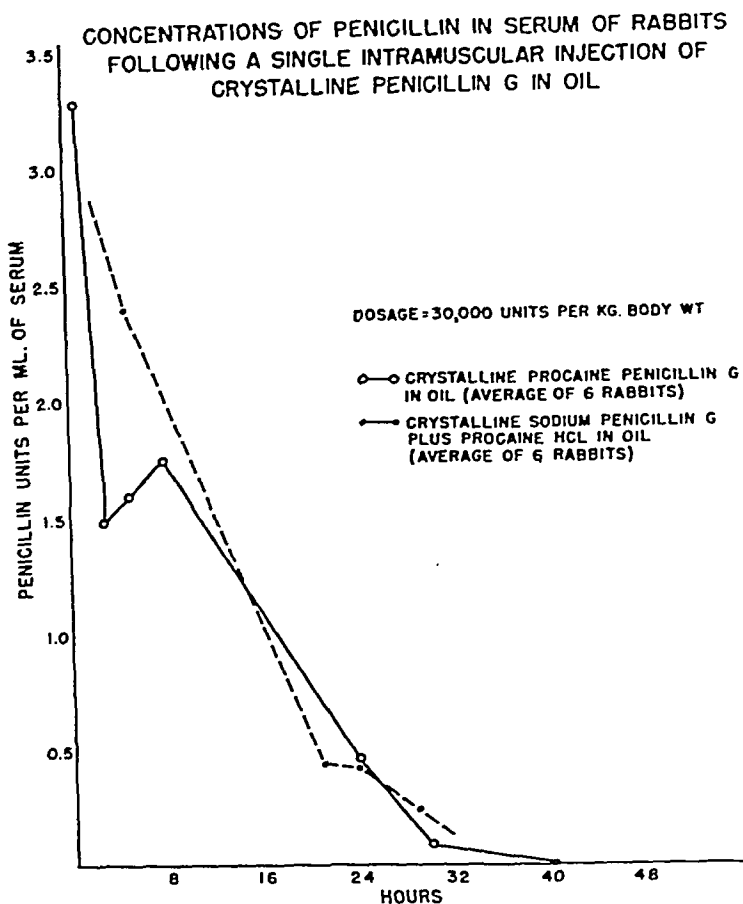


FIG. 1.

served with crystalline procaine penicillin dihydro-F.

The fact that certain impurities are capable of enhancing the action of crystalline sodium penicillin G has been discussed in previous communications.^{5,12} Preparations containing mixtures of crystalline procaine penicillins G and dihydro-F with certain of the naturally occurring penicillin mold pigments or other impurities have shown a similar enhanced action. Whereas 300 units of crystalline procaine penicillin G were capable of protecting 70% of animals against infection due to *Streptococcus hemolyticus*, this amount of the mixed procaine penicillins G and dihydro-F, with accompanying impurities, was capable

of protecting 90% of animals. One hundred and fifty units of these preparations of mixed crystalline penicillins were almost as effective as 300 units of crystalline procaine penicillin G or dihydro-F. The enhanced action of these mixed penicillins was not apparent in the small series of animals tested with pneumococcus.

Absorption and Excretion of Crystalline Procaine Penicillin. Crystalline procaine penicillins G and dihydro-F were prepared in a concentration of 300,000 units per ml peanut or sesame oil. Similar preparations of mixtures of crystalline procaine penicillins G and dihydro-F and accompanying impurities were also used. All experiments were carried out in normal male rabbits, weighing approximately 3 kg. In all instances 30,000 units per kg body weight were administered by the intra-

¹² Hobby, G. L., Lenert, T. F., and Hyman, B. *J. Bact.*, 1947, 54, 305.

CONCENTRATIONS OF PENICILLIN IN SERUM OF RABBITS FOLLOWING A SINGLE INTRAVENOUS INJECTION OF CRYSTALLINE PROCAINE PENICILLIN IN OIL

DOSAGE: 30,000 UNITS PER KG. BODY WT.

- CRYSTALLINE PROCAINE PENICILLIN G
- △ CRYSTALLINE PROCAINE PENICILLIN DIHYDRO-F
- ▲ MIXED CRYSTALLINE PROCAINE PENICILLINS G
AND DIHYDRO-F WITH PIGMENTS
- CRYSTALLINE SOLUBLE SALTS OF PENICILLIN G
(Na, NH₄, K, Li) + PROCAINE HCL.

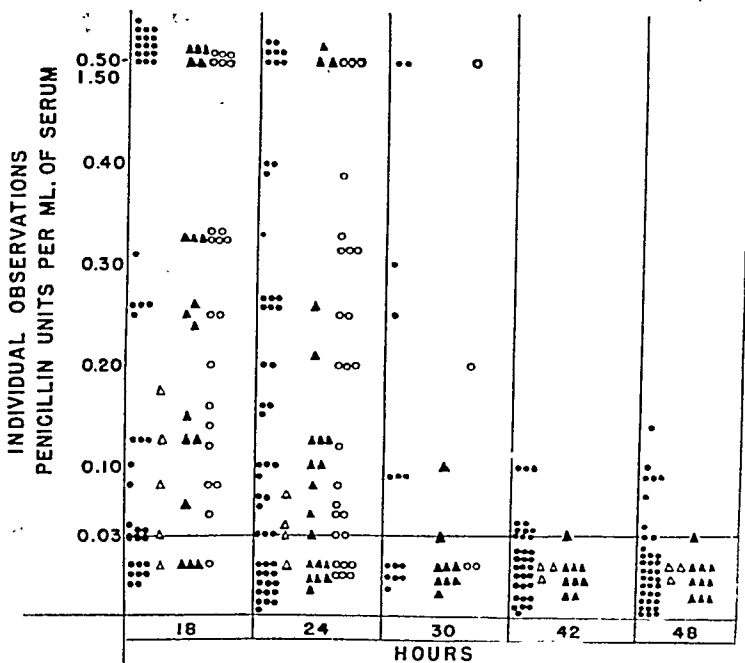


Fig. 2.

muscular route. Animals were bled immediately prior to and at varying intervals after injection. The concentration of penicillin in the blood was determined by the method of Tompsett, Schultz, and McDermott,⁴ using undiluted serum as well as serum diluted 1:4 and 1:20. *Streptococcus hemolyticus* was used throughout as the test organism and crystalline sodium penicillin G as standard. The minimum level detectable by this method varied from 0.02 to 0.04 units per ml. No attempt was made to determine the urinary excretion of penicillin from the rabbits injected.

As indicated in Fig. 1 and 2, a single

intramuscular injection of 30,000 units of crystalline procaine penicillin G in oil per kg of body weight produces in the majority of experimental rabbits detectable blood levels for periods of 24 to 30 hours. Blood levels ranging from 0.03 to 1.5 units per ml were ob-

|| In a few instances crystalline procaine penicillin G as well as sodium penicillin G was used as standard. The levels on this basis were at times slightly higher due to the fact that the sensitivity of the test organism (*Streptococcus hemolyticus*) to crystalline procaine penicillin G may be less than its sensitivity to crystalline sodium penicillin G.

ABSORPTION AND EXCRETION OF PENICILLIN IN MAN CRYSTALLINE PROCAINE PENICILLIN IN OIL

DOSE: 300,000 UNITS BY INTRAMUSCULAR ROUTE

— URINE

— SERUM

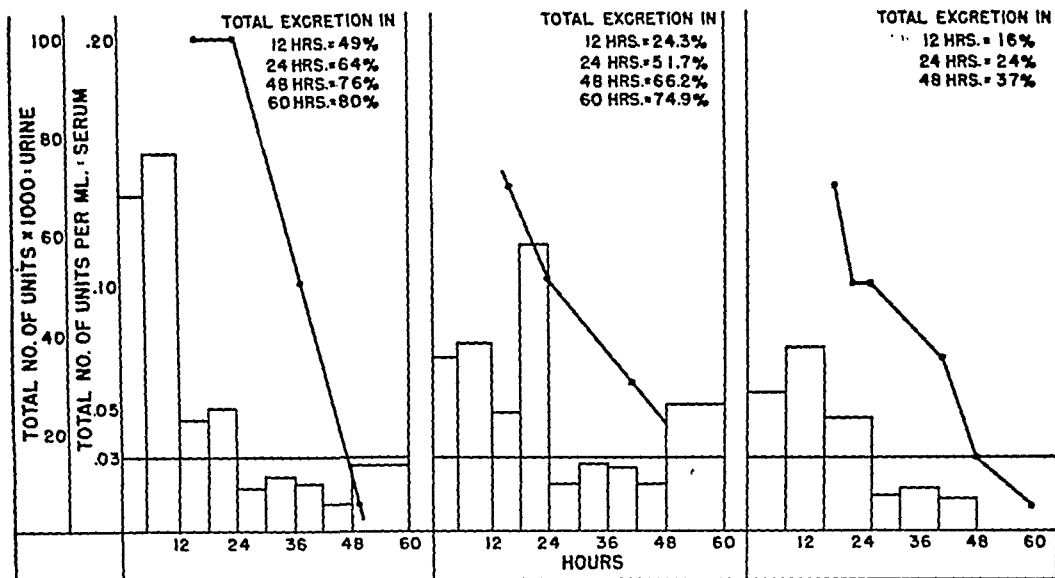


FIG. 3.

served in 80% of animals tested at 18 hours after injection. Sixty-seven per cent showed levels within this range at 24 hours, 50% at 30 hours, 33% at 42 hours, and 25% at 48 hours. Comparable results were obtained with crystalline sodium (potassium, ammonium, or lithium) penicillin G when mixed with procaine hydrochloride in oil in an amount equivalent to that present in crystalline procaine penicillin G. Likewise similar results were observed with other local anesthetics such as procaine buterate and procaine borate. Prolonged but lower levels resulted from the administration of crystalline procaine penicillin dihydro-F in oil, in the same dosage. The use of aqueous rather than oil suspensions of procaine penicillin gave less prolonged penicillin blood levels.

Comparative studies of crystalline potassium penicillin in oil and beeswax, using the same dosage and experimental conditions as those used for procaine penicillin, resulted in detectable blood levels lasting from 18 to 24 hours only.

Preliminary Observations on the Absorp-

tion and Excretion of Crystalline Procaine Penicillin in Man. Preliminary observations in man indicate that a single injection of 300,000 units of procaine penicillin G in oil will result in detectable concentrations of penicillin in the blood for periods of 24 to 48 hours, while high concentrations of penicillin may exist in the urine for at least 48 hours (Fig. 3).**

Toxicity of Crystalline Procaine Penicillin. Determination of the toxicity of crystalline procaine penicillins G and dihydro-F in animals is limited by the solubility of the compound. Solutions containing 6,000 units per ml have produced no toxic reaction when administered by the intravenous route to white mice in dosages of 1,400 units per 20 g mouse. Furthermore, rabbits receiving 4,000 units

** Since this paper was submitted for publication, a report has appeared by Herrell and his associates (Herrell, W. E., *et al.*, *Proc. Staff Meet. Mayo Clinic*, 1947, **22**, 567), who have administered procaine penicillin in oil to man in dosages of 300,000 units, and have observed prolonged blood levels similar to those reported herein.

CONCENTRATIONS OF PENICILLIN IN SERUM OF RABBITS FOLLOWING A SINGLE INTRAVENOUS INJECTION OF INSOLUBLE INORGANIC & ORGANIC SALTS OF PENICILLIN

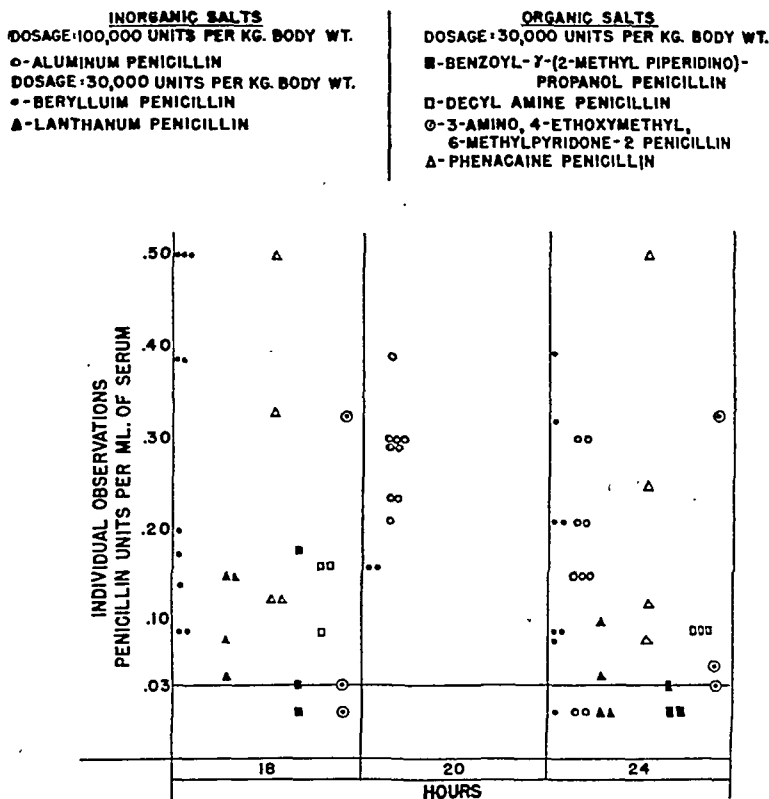


FIG. 4.

intravenously per kg body weight have shown no toxicity. Suspensions containing 40,000 units per ml have caused no reactions in dosages of 40,000 units per 20 g mouse when administered by the subcutaneous route.

It is recognized that the acute mouse toxicity of the soluble salts of penicillins is a direct measure of the concentrations of cation present. In like manner, the minimum lethal dose (LD_0) of crystalline procaine penicillin G (1400 units = 1.35 mg procaine penicillin = 0.56 mg procaine base) is in close agreement with that of procaine hydrochloride ($LD_0 = 0.60$ mg).

Preliminary observations in man indicate that procaine penicillin in oil is nonirritating on injection and produces no local pain or

soreness. It seems probable that crystalline procaine penicillin in oil possesses no greater toxicity than its component parts.

Preliminary Observations on Other Water-Insoluble Salts of Penicillin. Crystalline procaine penicillins G and dihydro-F are water-insoluble salts of penicillin. Their maximum solubilities have been found to be 6,700 units (6.5 mg) and 15,000 units (15.2 mg) per cc, respectively. In view of the fact that procaine penicillin in oil prolongs penicillin blood levels, it seemed likely that other insoluble salts of penicillin might act similarly. That this is true is indicated by the work of Monash¹³ who has recently reported detectable blood levels in rabbits at 18 and 24 hours following the intramuscular injection of silver

penicillate, mercury penicillate, and ferric penicillate in oil. Furthermore, Bohls and his associates¹⁴ have indicated that detectable blood levels may be attained for prolonged periods after the intramuscular injection of aluminum-penicillin in oil.

In the present investigation a small series of other water-insoluble organic and inorganic salts of penicillin was tested in experimental rabbits, using the procedure previously described. A dosage of 30,000 units per kg body weight was used throughout. The results are indicated in Fig. 4. Significant levels were observed at 18 and 24 hours in all instances. Beryllium penicillin, the least soluble of the compounds tested, showed the highest and most prolonged levels.

Discussion. Early studies¹⁵⁻¹⁷ on the bacteriostatic and bactericidal properties of the blood of animals treated with aqueous penicillin, as well as subsequent studies on the absorption and excretion of penicillin in man,¹⁸⁻²⁰ have indicated that penicillin remains in the blood stream for only 2 to 3 hours after injection. These observations have been amply confirmed by subsequent investigators during the past 4 years.

Although it was originally assumed that effective bacteriostatic concentrations of penicillin should be maintained constantly in the circulating blood, as was the case with the sulfonamides, it was soon recognized that 2 injections per day were sufficient for the control of certain experimental infections in animals.^{17,21} Furthermore, it was demonstrated by Tillett²² and more recently by Finland,²³ and by Tompsett and McDermott²⁴ that

pneumococcus pneumonia may be treated successfully with penicillin regimens which afford detectable levels in the blood during only a fraction of each day.

The preponderance of clinical experience with penicillin, however, has been obtained with dosage schedules which maintain continuous or nearly continuous measurable concentrations of penicillin in the blood throughout the period of treatment. For this purpose penicillin in oil and beeswax has been used to advantage.²⁵⁻²⁷ It has been amply demonstrated that a single injection of 300,000 units of penicillin in oil and beeswax will maintain detectable concentrations of penicillin in the blood for 12 to 24 hours; however, the reported irritation and hypersensitivity reactions following the administration of this form of penicillin have indicated the need for other methods by which prolongation of penicillin blood levels can be attained.

The fact that such prolongation may result from the use of water-insoluble salts of penicillin, suspended in oil, is of interest. Whereas the majority of these salts are only slowly absorbed, the possible toxicity of many of them limits their use. The toxicity of procaine has been reviewed by Graubard and his associates²⁸ in a recent communication dealing with the use of procaine intravenously in man. The toxicity of procaine injected intravenously varies with animal species. The minimal lethal dose in rabbits, guinea pigs, and dogs is reported to be in the vicinity of 40 mg per kg body weight; in man, the toxicity of procaine is thought to be dependent upon the

¹³ Monash, S., *Science*, 1947, **106**, 370.

¹⁴ Bohls, S. W., *et al.*, *Texas State J. Med.*, 1945, **41**, 342; *J. Ven. Dis. Inform.*, 1946, **27**, 69.

¹⁵ Chain, E., *et al.*, *Lancet*, 1940, **2**, 226.

¹⁶ Abraham, E. P., Florey, H. W., *et al.*, *Lancet*, 1941, **2**, 177.

¹⁷ Hobby, G. L., Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 277.

¹⁸ Rammelkamp, C. H., *et al.*, *J. Clin. Invest.*, 1943, **22**, 425.

¹⁹ Kirby, W. M., *et al.*, *J. Clin. Invest.*, 1944, **23**, 789.

²⁰ Dawson, M. H., and Hobby, G. L., *Ann. Int. Med.*, 1943, **19**, 707.

²¹ Dawson, M. H., and Hobby, G. L., *J. A. M. A.*, 1944, **124**, 611.

²² Tillett, W., *et al.*, *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

²³ Finland, M., personal communication, 1947.

²⁴ Tompsett, R., and McDermott, W., personal communication, 1947.

²⁵ Romansky, M., and Rittman, G. E., *Science*, 1944, **100**, 196.

²⁶ Romansky, M., and Rittman, G. E., *New Eng. J. Med.*, 1945, **233**, 577.

²⁷ Romansky, M., Murphy, R. J., and Rittman, G. E., *J. A. M. A.*, 1945, **128**, 404.

²⁸ Graubard, D. J., *et al.*, *N. Y. State J. Med.*, 1947, **47**, 2187.

TABLE II.
Chemotherapeutic Action of Crystalline Procaine Penicillin on Hemolytic Streptococcal (Group A) and Pneumococcal Infections in Mice.

Culture	Preparation of penicillin Total dosage in units	Procaine penicillin in oil			Potassium penicillin G in oil and beeswax	Untreated controls
		G	Dihydro-F	Mixed		
		Therapeutic effect: % survival				
<i>Streptococcus hemolyticus</i> (C230Mv)	600		75.0			
	450		69.4			
	300	70.0	67.4	90.0	83.3	
	150	51.7	30.0	63.3	43.3	
	50	23.4	20.0	45.0	23.4	8.8
<i>D. pneumoniae</i> (I/230)	500	95.0				
	400	80.0		70.0		
	300	72.5		77.5		
	150	65.0		42.5		
	50	30.0		20.0		

* A minimum of 10 to 15 mice was used for each dilution in each set. One ml of a 10⁻⁷ dilution contained 1-10 lethal doses of hemolytic streptococci or pneumococci; 10⁻⁶, 10-100; 10⁻⁵, 100-1,000; 10⁻⁴, 1,000-10,000. In all instances mice were injected intraperitoneally with 1 ml of 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions of culture.

percentage concentration administered.

The administration of penicillin in 1% procaine hydrochloride has been used frequently to eliminate local irritation and soreness following injection.²⁹ Crystalline procaine penicillin contains a high concentration of procaine (120 mg per 300,000 units). In preliminary studies it has produced little or no toxicity however.

That crystalline procaine penicillin is a highly efficient antibacterial agent both *in vitro* and *in vivo* has been demonstrated. Furthermore, the fact that this form of penicillin when suspended in oil will prolong blood levels in animals for 24 to 30 hours, or more, following the intramuscular injection of 30,000 units per kg has been shown. Preliminary observations in man indicate that a similar prolongation of blood levels occurs.

The mechanism by which procaine penicillin acts is not known. It is probable that the action is dependent upon at least 3 factors: (1) the low solubility of procaine penicillin in aqueous fluids, (2) the protect-

ive action of the oil surrounding the particles of procaine penicillin, and (3) the pharmacological activity of procaine on the tissues at the site of injection.

Conclusions. Crystalline procaine penicillins G and dihydro-F are highly effective antibacterial agents *in vitro* and *in vivo*.

Crystalline procaine penicillins G and dihydro-F, in oil, when injected intramuscularly in rabbits in a single dose of 30,000 units (0.1 cc) per kg body weight, in most instances produces blood levels lasting 24 to 30 hours or longer.

Preliminary observations in man indicate that a single intramuscular injection of 300,000 units of crystalline procaine penicillin G in oil may produce detectable blood levels lasting from 24 to 48 hours while penicillin may be excreted in the urine for at least 48 to 60 hours.

Preliminary observations suggest that the toxicity of crystalline procaine penicillin is probably low.

Other water-insoluble salts of penicillin when suspended in oil also produce marked prolongation of blood levels.

²⁹ Buckles, D. L., *Bull. U. S. Med. Dept.*, 1947, 7, 648.

Titration of Small Amounts of Mustard and Other Gases with Bromine and Methyl Red.*

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From the Rockefeller Institute for Medical Research, Princeton, N.J.

The quantitative determination of mustard gas and other war gases is difficult since very minute quantities must be analyzed. The method used should also be simple and rapid since it is often necessary to analyze a large number of samples. Mustard gas and the arsenic gases are known to react rapidly with bromine, a reagent which allows extremely accurate titration in very dilute solutions. The usual starch-iodide indicator is not sufficiently accurate for titration with 10^{-4} molar bromine and no satisfactory indicator has been reported.

A number of different oxidizing agents and different indicators have been tried but bromine and methyl red are the most satisfactory in 10^{-4} M solution. Hypochlorite solution may be used in place of bromine. It is much more stable, but reacts with many compounds with which bromine does not.

The method finally developed determines mustard gas, Lewisite, and ethyl or phenyl dichlorarsine within an error of ± 0.02 ml 10^{-4} molar bromine per ml final volume of the titrated solution. It should be useful for the titration of any compounds which react rapidly with bromine in acid solution.

1. Description of Samples of Gases Tested.

Mustard (I)—1 technical, prepared by Levinstein method (Sartori, M., *The war gases*, New York, D. Van Nostrand Co., Inc., 1940, 223) and redistilled; one C.P. No difference noted.

Ethylchlorarsine (II)—one C.P. and one technical. Technical titrates slightly lower.

Lewisite (III)—2 technical samples.

Phenyldichlorarsine (IV)—one technical sample.

2. *Preparation of Solutions.* Weighed or measured amounts of I, III, and IV were dissolved in alcohol to give a 10^{-2} M solution. From these, 10^{-4} M solutions were prepared by dilution with water. Solutions of III and I were also prepared in water alone in the same way as those prepared from 10^{-2} M in alcohol.

A 10^{-4} M solution of II was prepared by addition of the gas to water, since II is partially oxidized in alcohol.

These aqueous solutions are referred to as "solutions of the gases," although actually they are solutions of the hydrolysis products of the gases.

3. *Bromine Solution.* Saturated stock solution: 50 ml one M hydrochloric acid is shaken with 10 ml liquid bromine and the solution is kept in a dark glass bottle in the presence of excess bromine. This solution is 0.5 M. The concentration remains constant.

Standardization of bromine solution for titration: When the saturated solution is diluted to 10^{-4} M there is usually a loss of 10-30% in strength due, presumably, to traces of reducing substances in the acid or water used. It is necessary, therefore, to standardize the dilute bromine. This may be done by titration against a solution of 10^{-4} M mustard gas or thiodiglycol in water, or with potassium iodide and 10^{-3} M thiosulfate in the usual way. Ten ml dilute bromine in one N sulfuric acid is added to one ml 10^{-2} M potassium iodide and the liberated iodine titrated with 10^{-3} M thiosulfate, with starch as indicator. One ml 10^{-3} M thiosulfate is equivalent to 0.5 ml 10^{-3} M bromine.

An approximately 10^{-4} M bromine solution was obtained by diluting the saturated bromine solution 1/4,000 with the 0.5 M sulfuric acid used in this work. This dilute bromine solution remains constant within 10% for 24

* This paper is based on work done for the Office of Scientific Research and Development under Contract OEMsr-129 with the Rockefeller Institute for Medical Research.

TABLE I.

Titration of the Various Gases in 0.1 M Sulfuric Acid with Methyl Red and 10^{-4} M Bromine.
Initial volume 1 ml.

Gas	Quantity, ml 10^{-4} M	ml 10^{-4} M bromine Corrected for blank
—	0	0.05 (blank)
I	0.1	0.11
	0.3	0.30
	1.0	0.95
	3.0	3.1
III	0.1	0.11
	0.3	0.31
	1.0	1.00
	3.0	2.95
Aqueous sol.—II		
II	0.1	0.09
	0.3	0.33
	1.0	0.96
	3.0	3.10
10 ⁻⁴ M aqueous sol. prepared from 10 ⁻² M EtOH sol.		
IV	0.1	0.09
	0.3	0.31
	1.0	1.05
	3.0	2.95

hours if kept in dark glass-stoppered bottles. It loses strength rapidly in strong light or if left open. For this reason the burette should be refilled shortly before titrating.

4. *Methyl Red Solution.* Five mg per liter in water.

5. *Concentration Range and Accuracy.* All of the determinations have been carried out with samples of 5 ml or less of a 10^{-4} M solution of the gas titrated with 10^{-4} M bromine, delivered from a 5 ml burette graduated to 0.02 ml. Under these conditions the titrations are accurate to about ± 0.02 ml of 10^{-4} M bromine per ml final volume of solution, i.e., if the volume at the end of titration is 2 ml the end point is sharp to ± 0.04 ml. If the final volume is 10 ml the end point is sharp to ± 0.20 ml. If larger quantities of gas are to be titrated stronger bromine solutions should be used.

Technique of the Determination. A sample of one to 5 ml of the solution is put into a 1.5 x 15 cm test tube and adjusted to approximately 0.1 M sulfuric acid by addition of a few drops of concentrated sulfuric acid. One drop of methyl red is added for each ml of solution. Dilute (10^{-4} M) bromine solution

is run in from the burette until the solution becomes nearly colorless. Another drop of methyl red is introduced and bromine added again until the solution is colorless. The end point can be judged best by holding a tube of water alongside the tube of solution which is being titrated. Both tubes should be allowed to rest vertically on white paper and observed from an angle of about 45° to the vertical. The end point is not reversible so care must be taken not to overstep. It is preferable to add the methyl red in two steps owing to partial irreversible oxidation of the indicator by local excess bromine during the titration. Determination of the end point is greatly facilitated by the use of a Fisher fluorescent titration lamp (Fisher Scientific Co., Pittsburgh, Pa.).

The concentration of sulfuric acid or methyl red may vary $\pm 50\%$ without affecting the results.

Results. Examples of titrations carried out as described are shown in Table I.

Sources of Error. Any substance which reacts with bromine will evidently interfere with the titration. Hydrogen sulfide and sulfides in general are the most common such substances.

Since rubber tubing and stoppers contain large amounts of sulfides the solutions must not come in contact with them.

Hydrogen Sulfide in Air. Hydrogen sulfide is not absorbed in one M sulfuric acid so that in analyzing samples of air, hydrogen sulfide is not titrated. It may be removed from acid solution by boiling for several minutes. Old sulfide solutions, however, cannot be freed

of reducing substances in this way.

Summary. Mustard (I), ethyldichlorarsine (II), Lewisite (III), and phenyldichlorarsine (IV) may be titrated with 10^{-4} M bromine using methyl red as indicator. The method is accurate to about ± 0.02 ml 10^{-4} M bromine per ml of solution titrated. The reaction of these compounds is that of one mole of bromine per mole of gas.

.16189

Carbonic Anhydrase in the Pallium of Primates Compared with that of Lower Mammals.*†

WINIFRED ASHBY.

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Carbonic anhydrase reversibly catalyzes the change of carbon dioxide and water to carbonic acid. It occurs in the central nervous system in amounts ranging from a doubtful positive in the cord of the hog¹ to a content in either the cerebellum or cerebrum approximately equal to one-tenth of that found in the blood.² None has been found in peripheral nerve of man and dog (unpublished). The content in the spinal cord of mature animals in which it occurs in any considerable amount is well below that of the medulla, pons and higher centers.³

Previously reported data indicated a well defined difference in the pattern of the quantitative distribution of carbonic anhydrase in the pallium of the lower animals studied, the dog, the hog and the cat, from that found in man. In the lower animals the cortex held the greater content. In man the pattern was

reversed; the white matter immediately below the cortex had a markedly greater enzyme content than the cortex. Exception was found in the motor cortex where more definite approximation to the animal pattern appeared in the leg area.⁴

The present study is an extension of work previously reported. The brains of 4 Rhesus monkeys were examined to see whether the pattern of man is found also in these primates; and additional data on other of the lower mammals, the horse, the steer, the sheep and the rabbit are given. Data on the guinea pig are not included as the subcortical white matter of the brain was too small in amount to be separated successfully from the cortex.

Technique. The technique employed for the determination of carbonic anhydrase was essentially that of Philpot and Philpot⁵ as modified by Keilen and Mann.⁶ Specific procedures employed⁴ and the method used to determine the enzyme activity attributable to the blood in the tissue⁷ are given elsewhere.

* This work was aided by a grant from the Supreme Council, Thirty-third Degree, Scottish Rite, Masons and the Northern Jurisdiction, U. S. A.

† I am indebted to Dr. Anna Dean Dulaney of the Pathological Institute of the University of Tennessee for monkey brains.

¹ Ashby, W., *J. Biol. Chem.*, 1944, **152**, 235.

² Ashby, W., *J. Biol. Chem.*, 1943, **151**, 521.

³ Ashby, W., *J. Biol. Chem.*, 1944, **155**, 671.

⁴ Ashby, W., *J. Biol. Chem.*, 1944, **156**, 323.

⁵ Philpot, F. J., and Philpot, J. St. L., *Biochem. J.*, 1936, **30**, 2191.

⁶ Keilin, D., and Mann, T., *Biochem. J.*, 1940, **34**, 1165.

⁷ Ashby, W., and Chan, D. V., *J. Biol. Chem.*, 1943, **151**, 515.

TABLE I.
Comparison of Carbonic Anhydrase Content of Tissue from Exsanguinated and Non-exsanguinated Guinea Pigs.

Tissue	Carbonic anhydrase : Units per gram					
	Control			Exsanguinated		
	Total	Correction for blood content	Net	Total	Correction for blood content	Net
Cerebrum	(Pig A) 26.7	3.4	23.3	(Pig B) 25.0	0.02	25.0
Cerebellum	(" C) 40.4	3.0	37.4	(" D) 49.3	0.8	48.5
Frontal Pole	(" C) 20.0	3.4	16.6	(" D) 18.7	0.3	18.4

The brain was sampled while fresh—preferably unfrozen. Part of a gyrus was dissected from the pallium and pure white matter beneath the gyrus was taken. This was called "D." The gyrus was then sliced vertically to expose the layer of cortex surrounding the white matter within the gyrus. Approximately the outer half of the cortex was removed with a sharp blade. The remaining inner portion was separated from the white matter by cutting or scraping. These three latter samplings were designated "A," "B" and "C" respectively. (Fig. 1)

Effect of Perfusion. Since the first 3 monkeys received had been used to produce malarial antitoxin and had been bled and perfused when sacrificed until there was little evidence of blood in the brain, it was considered desirable to control the effect of perfusion by using guinea pigs. The degree of anemia produced in these animals was such that the carbonic

anhydrase attributable to the blood in the tissue was reduced from 3.0 units or more in the control animals to only 0.8 to 0.02 units in the perfused. The enzyme content of the brain tissue, however, was within the same range in the two series. The results are given in Table I. There was no indication that carbonic anhydrase activity of the brain was lost by perfusion.

Man vs Rhesus Monkey. The carbonic anhydrase content found in the cerebrum of the 4 monkeys studied was low. The content in the cerebellum was 2 to 3 times greater and within the range found in man.

Although the difference between the carbonic anhydrase content of the white and grey matter in the pallium was less in monkeys than in humans, especially in the brains with the smaller amount of enzyme, the pattern of distribution was the one typically found in the human brain. No attempt was made to determine whether, as in the human, the motor cortex of these monkeys gave the reverse distribution typical of the dog, cat and hog. Results obtained by the same technician, from one human and 4 monkey brains are given in Fig. 2 in which the gyrus in the region from which the test material was taken is magnified.

Horse vs Man. Comparisons, in the brain of a horse and of a man, of the carbonic anhydrase content of the cortex with that of the white matter immediately below it, are given in Fig. 3.

In 3 samplings, taken from the parietal area and from the frontal and occipital poles of the brain of the horse, there was definitely less carbonic anhydrase in the white matter

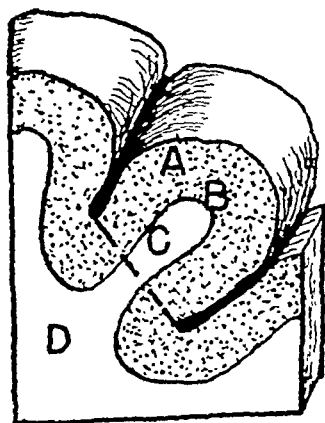


FIG. 1.

Illustrating the method used in dissecting the gyrus into the samples "A," "B," "C," and "D."

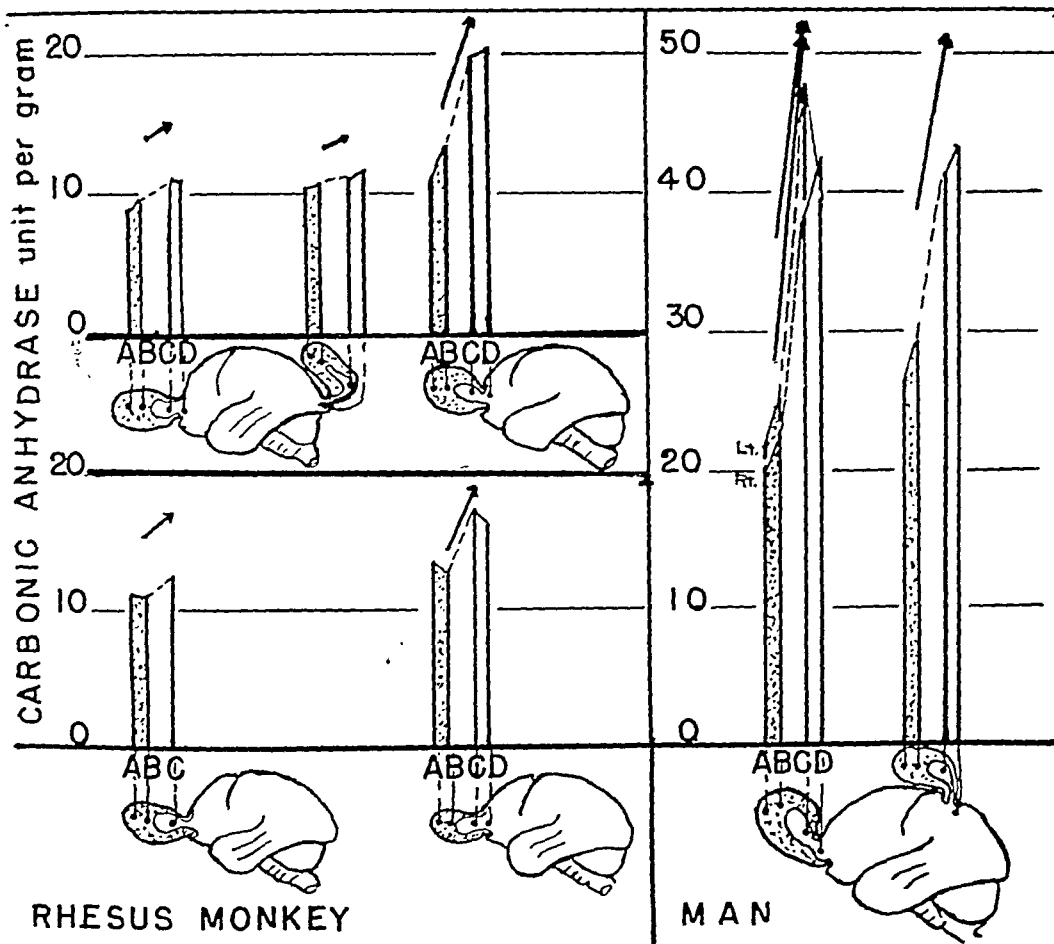


FIG. 2.

Pattern of distribution of carbonic anhydrase between cortex and subcortical white matter, in the cerebrum of a man and Rhesus monkeys.

"A," "B," "C," "D" have the significance indicated in Fig. 1. In the areas from which specimens were taken a gyrus is drawn as though greatly magnified and the "A," "B," "C" samplings are indicated thereon.

below the cortex than in the cortex. In the frontal pole the findings tended to be approximately equal in this brain, in marked contrast with the pattern found in man where, in the frontal pole, there was a great excess of enzyme in the white matter over that of the cortex and in the occipital pole, an approach to equality.⁸ The pattern of distribution found in the motor cortex of the human brain was as previously reported, like that found in the horse and in other non-primates studied.

Studies on Sheep, Rabbits and Cattle. Data on the relationship between the carbonic anhydrase content of the cortex and of the white matter below it, in the brains of 2 sheep, 3 rabbits, a calf and a steer, are given in Table II. Sheep I was sacrificed because of its extreme age. Sheep 2 was comparatively young. The rabbit brains were too small to make more than one series of determinations per brain. The calf had been exsanguinated. The steer was an experimental animal and had not been bled. Since its blood was not available, the blood of a cow was used to determine the correction to be made for

⁸ Ashby, W., *J. Biol. Chem.*, 1944, 150, 331.

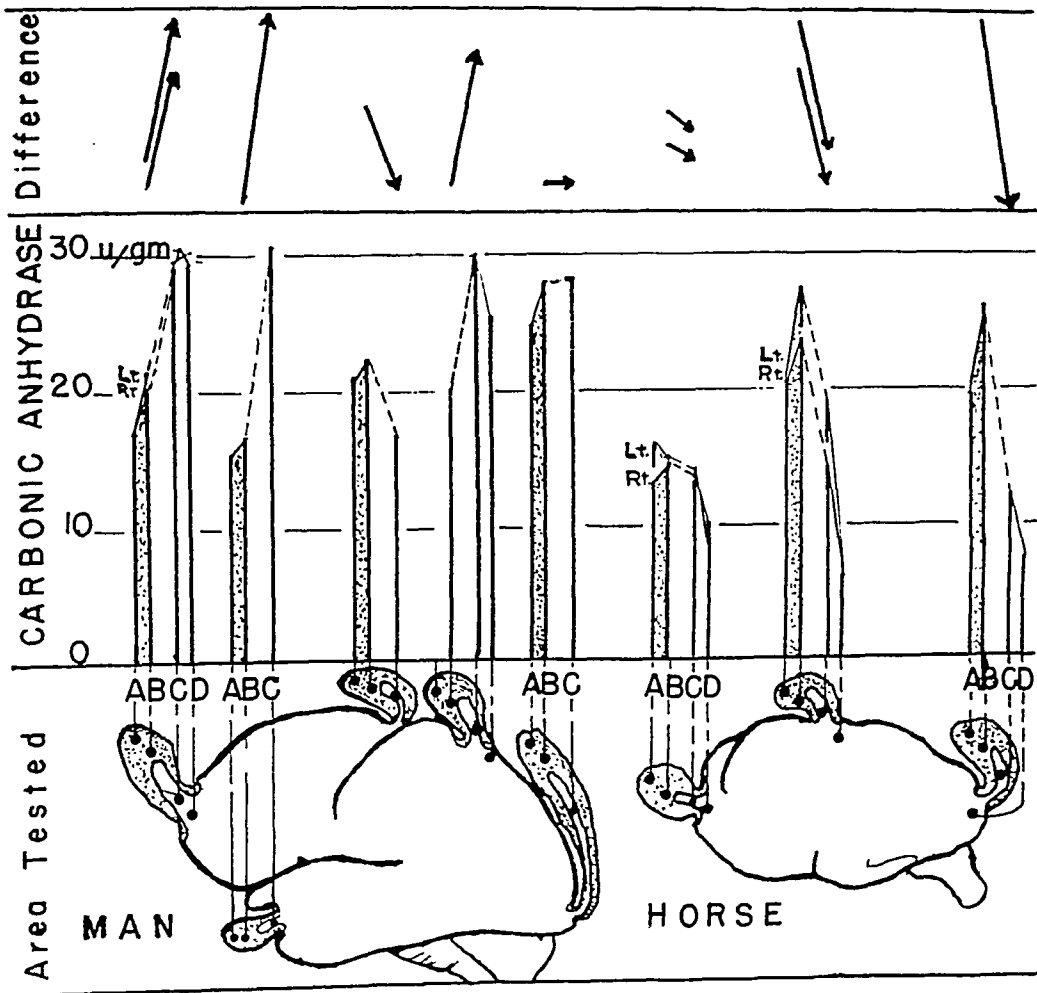


FIG. 3.

Distribution pattern of carbonic anhydrase in the pallium of a man and a horse. In man data on the leg motor area is included. "A," "B," "C," and "D" have the significance indicated in Fig. 1.

blood content. It is probable that the correction in the cortical specimens was too great (Table II).

Summation of Average Results. The data on the relative carbonic anhydrase contents of the cortex and the subcortical white matter are summarized in Table III and compared with findings from 10 frontal and 10 occipital poles from the same human brains. Averaged results from motor areas are also given. Subtraction of the average carbonic anhydrase content of the white matter immediately below the grey matter

from that of the grey matter gives a positive number in all species below the primates. In man and Rhesus monkey, on the other hand, a negative quantity is found except in the motor area.

Discussion. It would seem probable that the quantitative pattern of distribution of carbonic anhydrase found in the pallium of man and Rhesus monkey and, so far, not in other animals studied (namely, the dog, hog, cat, horse, cattle, sheep, rabbit) may be part of an evolutionary development peculiar to the primates.

TABLE II.

Comparison of Carbonic Anhydrase Content of Cortex with That of Sub-cortical White Matter in Sheep, Rabbit, and Cattle.

Animal	Area	Carbonic anhydrase, units per gram, corrected for blood content: correction in parentheses			
		Cortex		Sub-cortical white	
		A	B	C	D
Sheep 1.	Frontal	17.1 (1.1)		16.3 (0.7)	
	Occipital	18.2 (0.9)		12.2 (0.7)	
" 2.	Frontal	38.2 (0.9)		19.0 (1.4)	15.8 (0.7)
	Precentral	34.0 (4.5)		18.0 (2.4)	
	Postcentral	39.0 (2.7)		16.6 (1.4)	
	Occipital	39.0 (4.5)		25.2 (1.8)	14.7 (1.4)
Rabbit 1.		16.8 (1.3)		13.5 (1.8)	
" 2.		35.1 (2.0)	24.1 (1.5)	22.3 (0.5)	
" 3.		37.9 (5.6)	32.9 (5.6)	24.7 (3.5)	
Calf		12.5 (0.2)	12.4 (0.1)	8.5 (0.1)	7.0 (0.1)
"		11.1 (0.1)	11.4 (0.1)	8.1 (0.1)	
Steer		12.7 (8.0)	12.9 (6.5)	11.8 (5.0)	12.5 (1.4)
"		18.2 (5.1)	16.0 (4.9)	12.9 (4.0)	12.0 (1.6)
"		11.6 (6.7)	9.4 (5.0)	9.9 (1.6)	8.7 (1.3)

TABLE III.

Comparison of Carbonic Anhydrase Contents of the Cortex and the Sub-cortical White Matter in Primates and Lower Animals.

Animal	No. of comparisons	No. of animals	Average findings Units per gram		Difference %
			Cortex	White matter	
Rabbit	3	3	27.3	20.2	26
Steer	5	2	12.8	10.2	20
Sheep	4	2	30.9	18.0	42
Horse	6	1	20.5	14.9	27
Cat	6	4	39.2	24.9	36
Dog	6	2	33.7	14.4	57
Hog	9	5	34.8	26.4	24
Rhesus monkey	5	4	11.2	14.9	-33
Man					
Frontal pole	10		19.2	29.7	-55
Occipital pole	10		25.3	29.2	-15
Motor cortex	11		33.1	26.2	21

A tendency toward an inverse relationship between the size of the animal and the rate of metabolism of its brain has been reported.⁹ In the series studied, the brain of the horse, which most nearly approaches that of man in size, shows a cortical content of carbonic anhydrase somewhat below that in the human brain. Therefore the larger amount of enzyme found in the subcortical white matter of man as compared with that found in the cortex might be regarded as an

increase of the enzyme in the white matter beneath the cortex, rather than as a decrease within the cortex.

In the occipital pole in man, where there is a point to point projection from the geniculate nucleus, there is a decreased difference between the enzyme content of the cortex and the white matter beneath it. In the motor cortex, where the white matter consists largely of efferent fibers, the usual pattern of distribution peculiar to man and the Rhesus monkey is not present. Here, especially in the leg area, the white matter below the cortex

⁹ Page, I. H., *Chemistry of the Brain*. Springfield, Ill., Thomas, 1937.

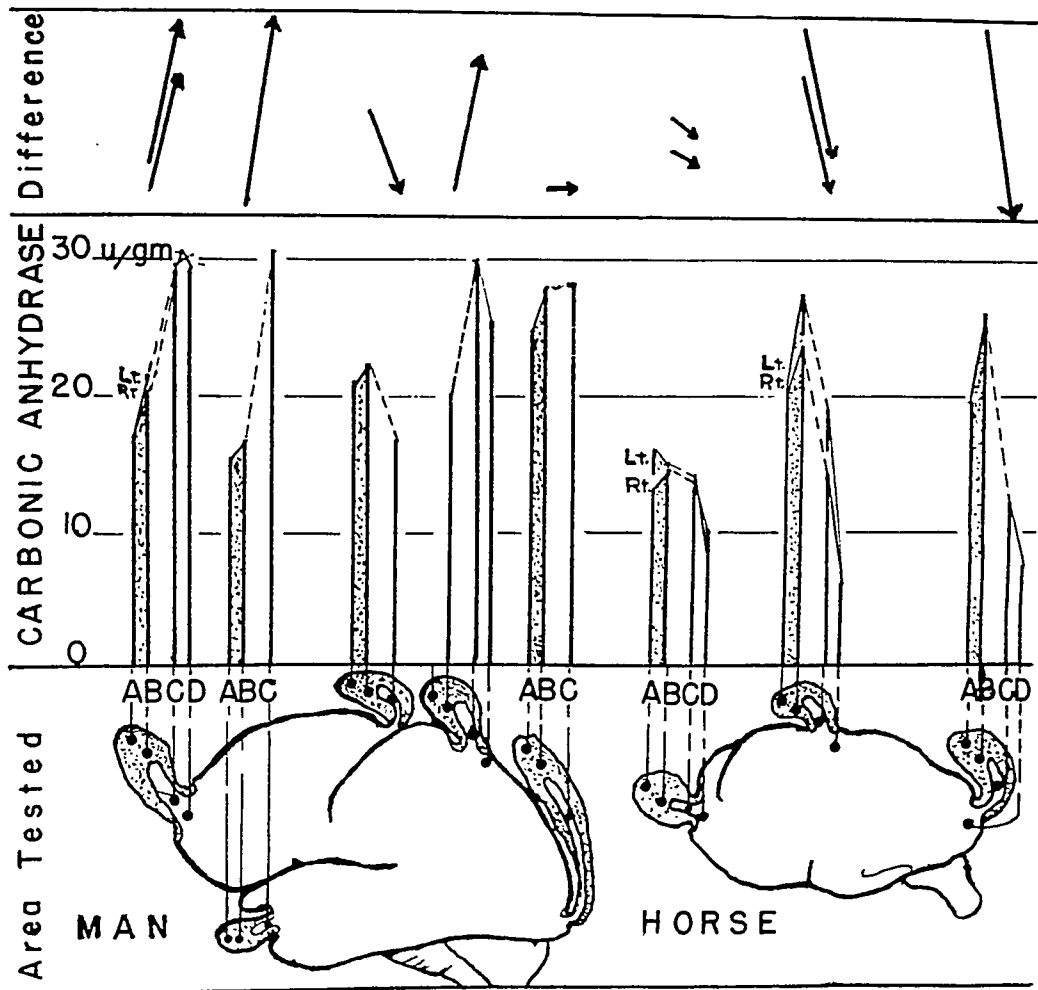


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Steer		12.7 (8.0)	12.9 (6.5)	11.8 (5.0)	12.5 (1.4)
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⁹ Page, I. H., *Chemistry of the Brain*, Springfield, Ill., Thomas, 1937.

contains definitely less enzyme than the cortex and the pattern of distribution is like that in the lower animals.

In this region below the cortex, which in the primates shows the increased enzyme content, are found fibers which make extrinsic cortico-cortical connections. The coincidence of this pattern of distribution with the development in the primates of the capacity for wider mental association, as found in man, may be of significance. If this difference in

the pattern of distribution proves to be tenable after further study, it would differentiate qualitatively between the brain of primates and that of the lower animals.

Summary. Within the pallium a quantitative distribution pattern of carbonic anhydrase, not found in the dog, cat, hog, horse, sheep, cattle and rabbit, has been found in man and the Rhesus monkey. Its possible significance is discussed.

16190

Purification of the Resin Amberlite IR-100 for Blood Coagulation Studies.*

MARIO STEFANINI.[†] (Introduced by Armand J. Quick.)

From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis.

The phenol-formaldehyde resin, Amberlite IR-100, which can remove calcium from blood completely by virtue of its ion exchange property¹ has become a valuable agent for quantitative studies of calcium in the coagulation mechanism.^{2,3} Since certain lots of Amberlite have been found to contain impurities which interfere with coagulation, an improved method of purification had to be devised. Several studies have been carried out with the purified product to determine further whether Amberlite induces any change in blood other than the removal of calcium.

Experimental. 1. Purification of Amberlite.[‡] Twenty grams of Amberlite were covered with 100 cc of 5% (by volume) sulfuric acid and

the mixture boiled and stirred vigorously for 5 minutes. The resin was washed with distilled water by decantation until the wash water was no longer acid. The material was then treated with 100 cc of 5% sodium carbonate solution, heated to approximately 70°C and thoroughly stirred. The deeply colored supernatant fluid was poured off and the process repeated until little coloring matter could be extracted. The resin was finally washed with warm distilled water until all traces of sodium carbonate were removed.

The cation-exchange resin was prepared for reaction by adding to it 250 cc of 5% solution of sodium chloride, stirring vigorously for 30 minutes and allowing the mixture to stand for 60 minutes more. The resin was then washed with distilled water and filtered by suction until the wash water no longer contained chloride ions. The solid was dried at 37°C.

2. Decalcification with Amberlite. To prevent incipient coagulation, blood obtained by venipuncture with minimal trauma was drawn into a syringe coated with Silicone and immediately passed through a column of 3 g of Amberlite, an amount sufficient for complete decalcification of 10 cc of blood. The

* This work was supported by a grant from the United States Public Health Service.

[†] Department of Internal Medicine, University of Roma; at present, Senior Research Fellow, National Institute of Health.

¹ Steinberg, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, 50, 124.

² Quick, A. J., *Am. J. Physiol.*, 1947, 148, 211.

³ Stefanini, M., and Quick, A. J., *Am. J. Physiol.*, in press.

[‡] Mr. James C. Winters of the Resinous Products and Chemical Company offered valuable suggestions.

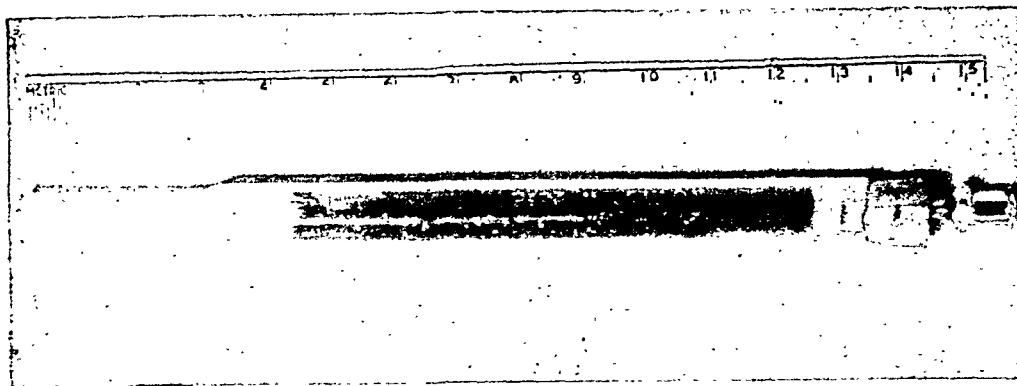


FIG. 1.

Tube Employed for Decalcifying Blood with Amberlite. The tube, the plug of glass wool, and the needle (No. 16) were all coated with Silicone; the cork with collodion.

tube used was approximately 1 x 15 cm and was coated with Silicone⁹ (Fig. 1). The decalcified blood was collected in a test tube coated with Silicone, then passed twice more through the same Amberlite. This resin can be re-used several times if it is washed free of all traces of blood with distilled water immediately after each use, then treated with 5% sodium carbonate and recharged with sodium chloride.¹

Properties of the blood decalcified with Amberlite. Steinberg's original findings¹ that the blood was little altered, except by removal of calcium, were confirmed. If any calcium remained, the amount was too small to be determined by ordinary analytical methods. Since dog blood thus decalcified failed to clot after the addition of thromboplastin, the calcium remaining must have been considerably less than 0.0001 M, a concentration still adequate to produce rapid clotting.² The thromboplastin used in this experiment and in all of our determinations of prothrombin time of plasma decalcified with Amberlite was made calcium-free. This was done by careful trituration of the rabbit brain from which it was prepared, with 0.02 cc of a 0.1 M solution of sodium oxalate per g of fresh weight, before dehydration with acetone.

Curiously, the sedimentation rates determined with blood decalcified with Amberlite

were consistently lower than with citrated or heparinized blood (Table I), thus confirming the findings of Steinberg. This observation may ultimately help to explain the sedimentation behavior of blood.

The clotting time of whole blood decalcified with Amberlite and recalcified with an optimum concentration of CaCl_2 (0.00386 M) appears to be much shorter than that of the original blood. For example, the clotting time of 8 minutes 15 seconds for a sample of native human blood was reduced by treatment with Amberlite and recalcification to 3 minutes and 30 seconds. However, a similar result is obtained with blood recalcified after oxalation or citration.

As shown by Steinberg, little or no modification of the prothrombin activity, as measured with the one stage method, takes place in blood decalcified with Amberlite; the fibrinogen content is slightly decreased (10-15%) but not sufficiently to cause any change in the coagulability. Human plasma that has been decalcified with Amberlite after the removal of prothrombin and fibrinogen can restore to normal the apparently delayed prothrombin time of stored human oxalated plasma.

The antithrombin activity of Amberlite plasma was compared with that of citrated or oxalated plasma by two methods. In the first, thrombin of decreasing strength was added to a constant amount of oxalated or Amberlite plasma according to the method

⁹ Methyl-chloro-silane; Dri Film No. 9987 (General Electric).

TABLE I.
Sedimentation Rate of Citrated, Heparinized, and Amberlite Human and Dog Blood After 1 and 24 Hours.
Westergren's technic was used. Values given are in mm.

Subject	Human blood				Dog blood			
	1	2	3	4	1	2	3	4
After 1 hr								
Citrated blood	10	14.5	23	17.5	2	2	2.5	3
Heparinized "	9.7	15	24	18	2	2	3	3
Amberlite "	4	9	15	10.5	1	1	1	2
After 24 hr								
Citrated blood	57	73	99	91	11	15	22	24.5
Heparinized "	58	75	102	94	10	16	20.5	26
Amberlite "	49	64	71	62	6	10	13	14.5

TABLE II.
Antithrombin Activity of Oxalated and Amberlite Plasmas of Man, Dog, and Rabbit.
Each figure in the table represents the average clotting time in seconds obtained in several experiments.*

Dilution of thrombin		Full strength	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
Human plasma	Amberlite	3	4	6	9	15.5	27
	Oxalated	3	4	6.5	9.5	17	29
Dog "	Amberlite	3	4	6.5	12	17.5	32
	Oxalated	3	4	7	11.5	19.5	35
Rabbit "	Amberlite	3.5	4.5	7	12.5	20.5	38
	Oxalated	3.5	5	7.5	13	22	39

* 0.1 cc of thrombin was added to 0.2 cc of the plasma. The tubes were incubated in a water bath at 37°C. The thrombin was always prepared from human oxalated plasma and stored for an hour or longer, until its activity became constant. It was diluted as required with distilled water.

of Quick.⁴ In the second, an equal volume of Quick's "full strength" human thrombin⁵ was incubated with Amberlite, oxalated or citrated plasma and the rate of decrease in thrombin activity was measured using normal oxalated plasma as a source of fibrinogen. Details are appended to the tabulated data (in Tables II and III).

The results recorded in Table II show plainly that the clotting response of Amberlite plasma to varying concentrations of thrombin is the same as that of oxalated plasma. Therefore, the Amberlite does not introduce or reduce any agent antagonistic to thrombin, nor does it affect the stability of fibrinogen. When thrombin is incubated with Amberlite plasma (Table III), its inactivation is less pronounced than when it is incubated with

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Summary. 1. A method of purification of resin Amberlite IR-100 was devised. When blood was treated with the purified resin a decreased sedimentation rate was found, with morphological, chemical, and physical properties otherwise practically unmodified.

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⁴ Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Charles C. Thomas, Springfield, Ill., 1942, p. 319, 321.

⁵ Quick, A. J., *Am. J. Physiol.*, 1936, 115, 317.

⁶ Quick, A. J., *Lancet*, 1947, 2, 379.

TABLE III.

Effect of Incubation with Citrated, Oxalated, and Amberlite Plasmas of Man, Dog, and Rabbit on Activity of Full Strength Human Thrombin.

Each figure in the table represents the average clotting time in seconds obtained in several experiments.*

Length of incubation (sec.)		60	120	180	240	300
Human plasma	Amberlite	8	11	13.5	29.5	34
	Citrated	12	14	16	21	29
	Oxalated	12	23	64	450	†
Dog	Amberlite	8.5	12	18.5	27	52
	Citrated	11	13	17.5	25	34
	Oxalated	13	26.5	49	90	225
Rabbit	Amberlite	9	11.5	14	22	40
	Citrated	11	12.5	15	20.5	34
	Oxalated	11	35	49	58.5	87

* Equal volumes of full strength human thrombin and of the plasma to be tested were incubated in a water bath at 37°C; the clot was wrapped about a glass rod coated with collodion and removed. After the incubation, 0.1 cc of the mixture were added to 0.2 cc of oxalated homologous plasma as a source of fibrinogen and the clotting time was determined and recorded.

† No clotting in 1 hr.

citrated plasma responded similarly, the behaviour may be linked with the mechanism of decalcification of the 3 agents.

16191 P

Effect of Polyoxyalkylene Sorbitan Monooleate on Blood Cholesterol and Atherosclerosis in Cholesterol-Fed Rabbits.*

AARON KELLNER,[†] JAMES W. CORRELL, AND ANTHONY T. LADD.
(Introduced by John G. Kidd.)

From the Department of Pathology, Cornell University Medical College, and The New York Hospital, New York City.

In an attempt to alter the solubility or colloidal stability of cholesterol *in vivo* and thus modify the development of experimental cholesterol atherosclerosis. Hueper¹ administered several commercial detergents to cholesterol-fed rabbits and observed no striking alteration in blood cholesterol levels or degree of atherosclerosis.

This report deals with the effect of oral polyoxyalkylene sorbitan monooleate, Tween

80,[‡] a surface-active agent of low toxicity, on the level of blood cholesterol and on the development of atherosclerosis in cholesterol-fed rabbits.

Experimental. Adult male and female rabbits averaging 3.2 kg in weight were fed a stock diet of Rockland rabbit pellets to which were added either:

A. Cholesterol—1 g; peanut oil—3 cc; Tween 80—10 cc.

B. Cholesterol—1 g; peanut oil—3 cc.

C. Tween 80—10 cc.

The ingredients were added separately to the stock diet in individual containers, and thoroughly mixed.

In Experiment 1, 6 rabbits were fed Diet A, and 5 Diet B daily. In Experiment 2, 12

* Aided by a grant from the United States Public Health Service.

† This work was done during the tenure of a Life Insurance Medical Research Fellowship.

¹ Hueper, W. C., *Arch. Path.*, 1944, **38**, 381.

‡ Manufactured by the Atlas Powder Co., Wilmington, Del.

TABLE I.
Sedimentation Rate of Citrated, Heparinized, and Amberlite Human and Dog Blood After 1 and 24 Hours.
Westergren's technic was used. Values given are in mm.

Subject	Human blood				Dog blood			
	1	2	3	4	1	2	3	4
After 1 hr								
Citrated blood	10	14.5	23	17.5	2	2	2.5	3
Heparinized "	9.7	15	24	18	2	2	3	3
Amberlite "	4	9	15	10.5	1	1	1	2
After 24 hr								
Citrated blood	57	73	99	91	11	15	22	24.5
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⁵ Quick, A. J., *Am. J. Physiol.*, 1936, **115**, 317.

⁶ Quick, A. J., *Lancet*, 1947, **2**, 379.

part at least, to better emulsification of cholesterol in the intestinal tract and more efficient absorption. Studies now in progress using parenteral administration of Tween 80 indicate that because of its detergent properties it may also have a more direct influence on the level of cholesterol and other lipids in the blood.

Rabbits fed cholesterol plus Tween 80 developed atherosclerosis of the aorta more uniformly, at an earlier date, and of a slightly more severe degree than the cholesterol-fed

controls. Animals fed only Tween 80 had no atherosclerosis.

Studies are now in progress to determine whether Tween 80 can effect resorption of experimental atherosclerosis.

Summary. Rabbits fed Tween 80 and cholesterol developed blood cholesterol levels that were 2 to 3 times as high as those obtained by cholesterol feeding alone, and also exhibited an earlier and somewhat more severe degree of atherosclerosis.

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V. Long-term Maintenance of Two Strains on Synthetic and on Stock Diets.*

PAUL F. FENTON, GEORGE R. COWGILL, AND MARIE A. STONE.

From the Nutrition Laboratory, Department of Physiological Chemistry, Yale University, New Haven, Conn.

Nielsen and Black¹ have reported that mice require a dietary source of biotin and folic acid during their early period of growth, and that this requirement is increased by incorporating sulfasuxidine in the ration. We² have observed that mice of 2 highly inbred strains grow well on synthetic rations containing no added biotin and folic acid. We have also shown, however, that one or both of these factors may be essential for satisfactory reproduction and lactation.³ Unpublished work in this laboratory has revealed that mice of the C₅₇ strain may be maintained on a synthetic diet with 0.75% sulfasuxidine without added biotin and folic acid but with p-aminobenzoic acid for about 6 months without show-

ing outward changes other than slight alopecia and achromotrichia. The discrepancy between our results and those of Nielsen and Black and the fact that the literature contains few reports of long-term maintenance of mice on highly purified diets led us to analyze the records of our mouse colony in which animals have been maintained on various stock diets and synthetic rations.

Methods. Two strains of mice (the A strain, with high incidence of spontaneous mammary tumors, and the C₅₇ with low tumor incidence) have been employed in these studies. At weaning the animals were placed in individual screen-bottom cages and their growth rates measured until they were 10 weeks old. Thereafter they were used for reproduction and lactation studies as previously reported.³ During the rest intervals between matings or after the end of the reproductive studies 2 or 3 animals were housed together. The stock rations employed were Purina Dog Chow and later Purina Laboratory Chow. The synthetic diet (No. 101)³ contained dextrose 60%, "vitamin-free" casein 23%, hydrogenated cottonseed

* This investigation was supported by grants from the Nutrition Foundation, Inc., the Anna Fuller Fund, and the American Cancer Society, on the recommendation of the Committee on Growth of the National Research Council.

¹ Nielsen, E., and Black, A., *J. Nutrition*, 1944, **28**, 203.

² Fenton, P. F., Cowgill, G. R., and Stone, M. A., *Yale J. Biol. and Med.*, 1947, in press.

³ Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **33**, 703.

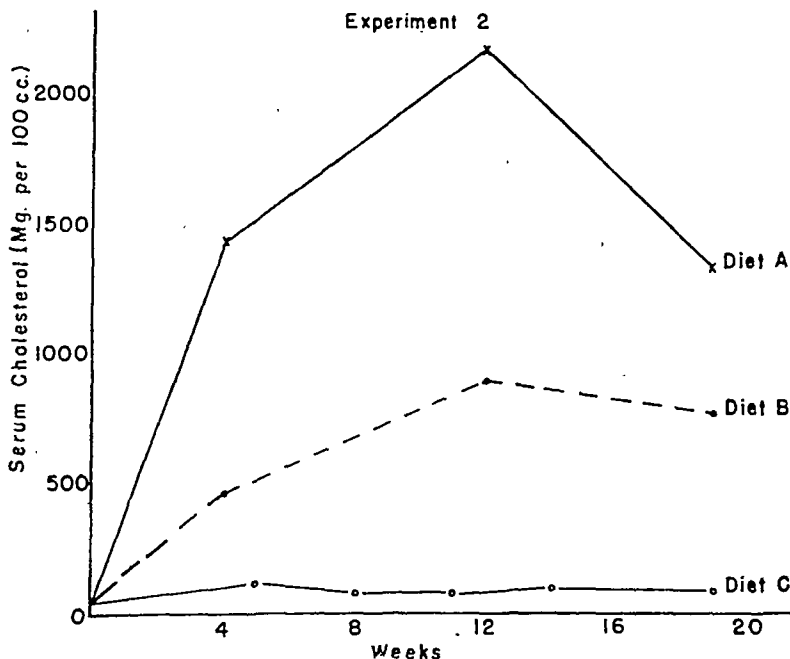


FIG. 1.

Mean Serum Cholesterol Levels.

Diet A—Cholesterol, peanut oil, and Tween 80 (12 rabbits).

Diet B—Cholesterol and peanut oil (11 rabbits).

Diet C—Tween 80 (3 rabbits).

rabbits were fed Diet A, and 11 Diet B 3 times a week, and 3 additional rabbits were fed Diet C daily. Blood was drawn from the marginal ear vein, and serum total cholesterol determined by a modification of the method of Bloor, Pelkan, and Allen.² Tween 80 *per se* does not influence the colorimetric determination of cholesterol, as it does not give the Leibermann-Burchard reaction itself or when added to serum *in vitro*. The degree of atherosclerosis of the aorta was determined by gross examination of the fresh specimen, and after fixation and staining of the entire aorta in Sudan IV.

Results. In Experiment 1, at 4, 7, and 10 weeks the mean serum total cholesterol levels of rabbits fed Diet A (cholesterol, peanut oil, and Tween 80) were 1,490, 2,725, and 2,150 mg per 100 cc respectively, as compared with 725, 975, and 1,160 mg per 100 cc for the rabbits fed Diet B (cholesterol and peanut

oil). Fig. 1 summarizes the results of Experiment 2. The serum total cholesterol levels reached a peak at 12 weeks with a mean value of 2,215 mg per 100 cc (range 900-4,350) for rabbits on Diet A, and 880 mg per 100 cc (range 285-2,205) for rabbits on Diet B. The difference between the two groups is more than 6 times the probable error, and is statistically significant. Rabbits fed Diet C (Tween 80) showed at first a very slight rise in blood cholesterol, which then returned to and remained within the normal range. The blood cholesterol levels of the rabbits fed Diet B were in approximately the same range as those previously reported in cholesterol feeding experiments (Weinhouse and Hirsch;³ Dubach and Hill⁴).

The action of Tween 80 in augmenting the blood cholesterol level is probably due, in

³ Weinhouse, S., and Hirsch, E. F., *Arch. Path.*, 1940, **30**, 856.

⁴ Dubach, R., and Hill, R. M., *J. Biol. Chem.*, 1946, **165**, 521.

² Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

Nielsen and Black did not incorporate p-aminobenzoic acid in their rations does not offer a convincing reason for the discrepancy. It seems much more likely that the difference is due to the nature of the dietary carbohydrate. We have used dextrose while Nielsen and Black used sucrose. This aspect of the problem is now being further investigated. Another possible explanation at the moment seems to be one of strain difference. Our evidence^{4,5} very strongly supports the view that there is a clear strain difference in the nutritional requirements of the C₅₇ and the A strain. The mice used by Nielsen and Black may have possessed such a high requirement for biotin and folic acid that they showed deficiency symptoms during a very early period of life.

The difference in the time required for A strain females to develop mammary tumors on the stock and the synthetic diets is highly suggestive and is being investigated more thoroughly.

The much greater incidence of symptoms

⁴ Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **34**, 273.

⁵ Fenton, P. F., and Cowgill, G. R., *Fed. Proc.*, 1947, **6**, 407.

and deaths of A strain mice on synthetic diets suggests again their greater nutritional requirements. We have shown in this laboratory^{4,5} that this strain requires more riboflavin and pantothenic acid for growth than does the C₅₇ strain. The findings reported here suggest also a greater need for biotin or folic acid or both in order to maintain the animal in good health during later life. The absence of possible unknown nutritional factors cannot be altogether overlooked. We have found⁶ that mice of the C₅₇ strain on synthetic diets had greater cecal contents and a greater bacterial population than did mice of the A strain. This strain difference was not observed in animals on stock ration.

Summary. Two strains of highly inbred mice, one tumor-susceptible and the other tumor-resistant, were maintained for a period of about one year on a stock diet or on a synthetic ration adequate for good growth performance. The tumor-susceptible A strain on synthetic diet showed numerous deficiency symptoms not seen on stock rations and not shown to any great extent by C₅₇ mice on either stock or synthetic diets.

⁶ Gall, L. S., Fenton, P. F., and Cowgill, G. R., in press.

16193 P

Antagonism of Sulfadiazine Inhibition of Psittacosis Virus by p-Aminobenzoic and Pteroylglutamic Acids.*

HERBERT R. MORGAN.[†]

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.

Studies on the chemotherapeutic action of the sulfonamides on certain bacteria¹ have shown that p-aminobenzoic acid (PABA) and pteroylglutamic acid (PGA) antagonize the inhibition of growth of these organisms by sulfonamide drugs. The demonstration of sul-

fadiazine (SD) inhibition of the growth of certain strains of psittacosis virus² suggests the likelihood that PABA and PGA might be active antagonists for this inhibitory effect of the sulfonamides.

A yolk sac passage strain of psittacosis

* Aided by a grant from the National Foundation for Infantile Paralysis.

[†] Senior Fellow in the Medical Sciences of the National Research Council.

¹ Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1947, **170**, 133.

² Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53**, 151.

TABLE I.
Summary of Observations.

Strain	Sex	C ₅₇				A			
		Male		Female		Male		Female	
	Diet No. of animals	Stock 14	101 8	Stock 30	101 11	Stock 9	101 19	Stock 21	101 28
Achromotrichia (slight)		2	1						
Alopecia (slight)			4	3	2	1	2		2
Skin ulceration							2		2
Eye involvement							6		1
Moist dermatitis					2		5		4
Massive fecal crusts							4		2
No. of animals with tumors								14	16
Avg time of onset—months								11½	10
Avg No. of litters born				4.3	2.7			4.0	1.9
No. of deaths									
Fecal crusts							4		2
“Tumors”								4	5
Incisor overgrowth					1				
Avg wt when sacrificed—g		28.2	30.3	29.0	26.8	27.8	25.2	—	—

oil 10%, salts 5%, roughage 2%, 8 vitamins of the B complex (thiamine, niacin, pyridoxine, riboflavin, pantothenate, choline, inositol, and p-aminobenzoic acid), cod liver oil concentrate, α -tocopherol, and linoleic acid. The animals on synthetic rations were given various supplements during the reproduction study;³ these, however, were only administered for relatively brief time periods. Because of lack of space, it was necessary to sacrifice most of the animals when they were about a year old.

Results. The pertinent observations made on these animals in the course of their existence or at the end of the year period are summarized in Table I. Mice of the A strain maintained on synthetic diet showed numerous symptoms which they did not develop on the stock ration. Most of these changes were not observed in the C₅₇ strain maintained on either diet. The eye lesions consisted of inflammation and denudation of the surrounding skin. Occasionally a dried exudate was observed in this area. Animals showing these conditions were usually hesitant to open their eyes when disturbed. The moist dermatitis which was observed almost exclusively in the A strain on synthetic diet was usually found below the chin and extended caudad for a distance of several centimeters. No hair was seen in the involved areas. Skin ulcerations, when present, were usually on the lateral body

areas and on the legs. The formation of massive fecal crusts over the anus was observed in 6 animals of the A strain on synthetic diet. This condition, which has always been fatal, has also been observed in the offspring of the generation of mice reported here. We have seen this happen only in A strain mice on synthetic diets. It has even occurred in the animals subsisting on the most complete synthetic diets which we have studied.

The causes of deaths in each group of animals are arranged in the table in 4 classes. When the cause of death is described as “tumor,” we mean only that the animals in question had a sizable mammary tumor which could have been the cause of death. No extensive autopsies were performed on the dead animals.

Discussion. We have not found under the conditions of our experiments that the mouse requires a dietary source of biotin or folic acid for early growth; nor have we observed any deficiency symptoms during the early period of life as did Nielsen and Black.¹ A possible explanation of this discrepancy may lie in the fact that Nielsen and Black carried out special alcohol extractions of their casein and sucrose, while we used commercial “vitamin-free” casein and dextrose C.P. without further treatment. Biotin assays on our diet 101 showed it to contain less than one μ g of biotin per 100 g of ration. The fact that

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Microbiological Determination of Apparent Free Methionine in the Blood of Children.

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Few studies on the blood levels of methionine, one of the amino acids indispensable for man, have been reported. By the use of the microbiological method of assay, Harper, Kinsell, and Barton,¹ found that the mean fasting level of apparent free *L*-methionine in the plasma of 11 normal male adults, was 0.85 ± 0.09 † mg per 100 ml with a range from 0.46 to 1.48. However, there has been no published information concerning the values in the newborn or older child. The purpose of this communication is to present the results of studies on the blood of (1) hospitalized "normal" children; (2) hospitalized children with various diseases; and (3) on the blood of the umbilical cord at the time of delivery.

Method. All of the children were in a post-absorptive state. Blood samples from them were obtained from either the antecubital or jugular vein. The "normals" had been hospitalized for the correction of non-infectious orthopedic difficulties. In the case of the newborn infants, cord blood was taken from the maternal end of the umbilical cord as soon as possible after delivery. Heparin was used as the anticoagulant in each case.

Proteins were removed from the plasma by heating and adding 5% acetic acid.¹ *L*-methionine in the filtrates was determined microbiologically, with *Leuconostoc mesenteroides* P-60 as the assay organism.² One ml of the basal medium recommended by Harper *et al.*¹ was used in each determination; the

plasma filtrates were added at 3 different levels (0.4, 0.6, and 1.0 ml); and the final volume was adjusted to 2 ml. All tubes were incubated for 72 hours and acid production was determined by electrometric titration with NaOH.

Results. The mean concentration of apparent free *L*-methionine in the plasma of "normal" children was 0.29 ± 0.02 mg per 100 ml of plasma. The range was from 0.19 to 0.47. The results are plotted in Fig. 1A. The difference between these levels and those found by Harper *et al.* in normal adult males with the same method of assay is statistically significant. Thirty-three hospitalized children, who were suffering from various diseases, had plasma methionine levels which were in the same range as those of the "normal" children. The mean was 0.42 ± 0.05 mg per 100 ml of plasma with a high of 1.07 and a low of 0.08 (Fig. 1B). At the time of testing, many of the children were convalescing, but there was no significant difference between their levels and those of the acutely ill ones.

Of 30 umbilical cord bloods tested, the mean level was 0.86 ± 0.04 mg per 100 ml of plasma (Fig. 1C). These results were in the same range as those found in adult males and were more than 3 times as high as those in "normal" children or those who had been ill.

Discussion. The foregoing data indicate that the plasma levels of apparent free *L*-methionine in the blood in the umbilical cord at birth is approximately the same as the fasting level reported for adult males.

At least two factors may operate to account for the lower plasma levels found in the children: (1) The main need for protein in the adult is to repair body tissue. The growing child needs an additional supply for growth and for the formation of new tissue. The increased demands in the child may lead to a greater rate of disappearance of essential

* With the technical assistance of Mrs. Tilly B. Leake.

¹ Harper, H. A., Kinsell, L. W., and Barton, H. C. *Science*, 1947, **106**, 319.

† Throughout this paper the figures following the symbol " \pm " refer to the standard error of the mean.

² Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, 1946, **163**, 577.

virus (6BC) was employed using the same experimental techniques previously described² for the determination of the effect of SD on the growth of the virus. PABA and PGA were prepared as sterile solutions of their sodium salts in distilled water. These solutions were mixed with the proper amounts of a sterile solution of sodium sulfadiazine just before injection in 0.25 ml amounts into the yolk sac of 7-day-old embryonated eggs. This was followed in $\frac{1}{2}$ hour by the injection of a yolk sac suspension of psittacosis virus diluted to contain 10,000 LD₅₀ in 0.25 ml. The doses of the various compounds are recorded in weights of PABA, PGA and SD.

Preliminary experiments have indicated that the minimal amounts of the two compounds required to antagonize the inhibitory effect of 2.5 mg SD on the growth of the virus was 0.005 mg PABA and 0.05 mg PGA. Following the establishment of these relationships, a series of experiments was carried out to determine the amounts of the 2 inhibitors required to antagonize the action of larger amounts of SD. Representative results are presented in Table I, using amounts of PABA and PGA twice and ten times the minimal doses noted above.

There is a direct relationship between the amount of SD used and the amount of PABA required to antagonize its inhibitory action. This suggests a competitive inhibition of the action of SD. In contrast, a given dose of PGA is demonstrated to be actively antagonistic for increasingly larger doses of SD which suggests a noncompetitive antagonism. These data suggest that, as is the case with certain bacteria which synthesize PGA,¹ the primary action of SD on psittacosis virus is directed against the incorporation of PABA into PGA by the virus because of the direct relationship between the amount of PABA required to antagonize a given amount of SD. When PGA is supplied, even large doses of SD fail to inhibit the growth of the virus. These findings suggest that psittacosis virus

TABLE I.
Antagonism of Sulfadiazine Inhibition of Psittacosis Virus by *p*-Aminobenzoic Acid and Pteroyl-glutamic Acid.

SD, mg	Inhibitor agent, mg	No. eggs	% survived 10 days*
0.5	PABA .01	7	0
2	" "	8	0
5	" "	6	0
10	" "	10	47
25	" "	13	85
50	" "	6	100
0.5	PABA .05	5	0
2	" "	8	0
5	" "	8	0
10	" "	18	0
25	" "	11	8
50	" "	4	100
0.5	PGA .1	7	0
2	" "	8	0
5	" "	6	0
10	" "	16	0
25	" "	11	0
50	" "	8	0
0.5	PGA .5	8	0
2	" "	7	0
5	" "	7	0
10	" "	20	0
25	" "	10	0
50	" "	7	0
0.5	—	16	82
5	—	24	96
—	PABA 5.0	8	0
—	PGA 10.0	10	0
—	—	16	0
—	Drug Controls.†	8	85
50	PABA 5	20	95
—	PGA 10	19	95

* 10,000 LD₅₀ injected into each egg.

† Not infected.

(6BC strain) synthesizes PGA and that this vitamin is required for its growth. Studies are now underway to elucidate this possible metabolic activity of the virus.

Summary. The chemotherapeutic action of sulfadiazine on psittacosis virus (strain 6BC) is antagonized competitively by *p*-aminobenzoic acid and noncompetitively by pteroyl-glutamic acid. The implications of these findings with regard to the metabolic activities of this virus are discussed.

Sarcomas Induced in Rats by Implanting Cellophane.

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From the Department of Cancer Research and the Department of Surgery, College of Physicians and Surgeons, Columbia University, New York.

In the course of experiments performed on rats to produce hypertension by wrapping regenerated cellulose film (cellophane) around one kidney, the accidental observation was made that several rats developed sarcomas in the neighborhood of the cellophane. As it was known that spontaneous sarcomas are a rarity in this Sherman strain of albino rats it was decided to investigate this question as an independent problem.

Method. The cellophane employed was regenerated cellulose film, the particular sample used throughout these experiments being known as Visking 5-1/2 H.S. ("high-stretch") cellulose sausage casing. Two strains of albino rats were used, the Sherman and the Wistar. The rats were fed on a mixture of Purina Laboratory Chow and Rockland Rat Diet in pellet form.

Cellophane was introduced by two methods into 110 male rats, 8 to 10 months old. In 55 of these, the left kidney was loosely wrapped with cellophane according to the familiar method introduced by Page.^{1,2} In the other 55 rats, a piece of cellophane 2-3 cm square was embedded subcutaneously in the abdominal wall, with a catgut suture passed through the corners to keep the film flat. At frequent intervals the site of the cellophane was palpated by an experienced technician to ascertain if any growth had started. The tumors were usually allowed to grow to a large size (2-5.5 cm in diameter) before the rats were sacrificed for histological examination.

Wherever possible, transplants were made from each tumor into 8-16 rats, usually males (but in some instances females), 6-8 months of age. A total of 422 rats were used for this

purpose, transplantation being carried out in some instances to the third, fourth and even fifth generation. Transplants were made by inoculating fragments of tumor tissue (0.003 to 0.006 g) subcutaneously into the anterior abdominal wall by means of a hollow platinum-iridium needle.

Results. As in the original accidental observation, a number of tumors were induced at the site of insertion of the cellophane. More than 11 months were required for their development. In the perirenal series the shortest time taken for the production of a tumor was 362 days from the date of wrapping to the development of a tumor 8 mm in diameter, whereas in the subcutaneous series the shortest period for development was 471 days.

Consequently, those rats that died from other causes within 11 months from the date of cellophane insertion were excluded altogether from the statistics, since they did not live long enough to develop induced tumors.

Among the rats with wrapped kidneys only 23 survived over 11 months, and of these 8 (34.8%) developed large well-defined tumors. Of the 55 rats in which the cellophane was embedded subcutaneously 42 survived beyond 11 months, and of these 15 (35.7%) presented tumors. The diagnosis of all these tumors was confirmed by microscopic examination.

The tumors resulting from transplantation appeared after 7 to 175 days. A total of 199 tumors were produced by transplantation from the primarily induced tumors, but this includes 126 instances in which well-defined growths subsequently receded, making microscopic examination impossible.

Types of Tumor. Of the 23 primarily induced tumors there were 17 fibrosarcomas, 2 liposarcomas, one rhabdomyosarcoma, one

¹ Page, I. H., *Science*, 1939, 89, 273.

² Graef, I., and Page, I. H., *Am. J. Path.*, 1940, 16, 211.

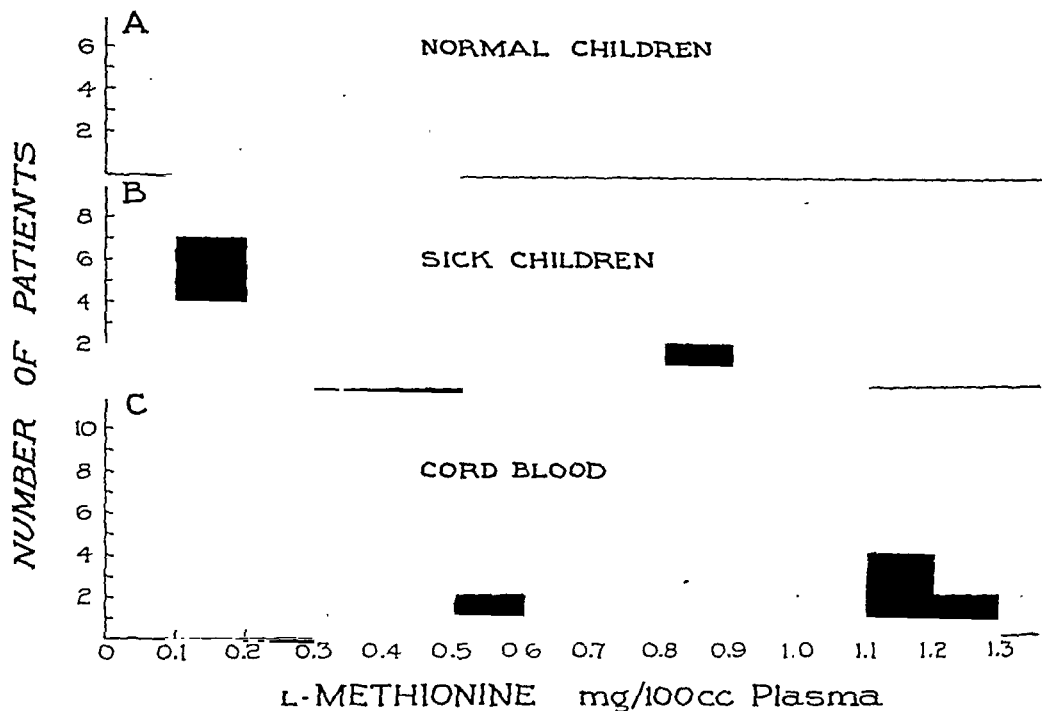


FIG. 1.

Apparent free *L*-methionine in plasma: (A) in hospitalized "normal" children; (B) in hospitalized children with various diseases; (C) in the blood in the umbilical cord at the time of delivery. The levels reported for normal adult males are 0.85 ± 0.09 mg per 100 ml of plasma.

amino acids from the blood. Hence, when an amino acid is determined in the plasma after a fasting period of 14-16 hours, less may be found in the child than in the adult. (2) A difference in the absorptive capacity of children and adults might play some part in making plasma levels of the former lower, but this seems unlikely. Preliminary tests in this laboratory, involving analysis of the blood of previously fasted subjects after the ingestion of 0.1 gm of methionine per kg of body weight, have shown that the amount of methionine absorbed in children approximates that found in adults.

Conclusion. The concentration of apparent free *L*-methionine in the plasma of children is significantly lower than has been recorded in the literature for adult male subjects. The plasma of the blood in the umbilical cord at the time of delivery contains, on the average, the same amount of *L*-methionine as is recorded for adult males, and more than three times as much as is present in the plasma of "normal" children.

Sincere thanks are expressed to Doctor Harold Harper, University of San Francisco, for helpful suggestions.

TABLE I.
Data for Several Characteristics of Gastric Pouch Mucus.
(Stimuli: aqueous emulsions of clove oil and eugenol.)

		Stimuli			
		Eugenol			Clove oil, (5%)
		Low concentration ($\frac{1}{4}$ - $\frac{1}{2}$ %)	Intermediate concentration (1-2%)	High concentration (5%)	
Volume per exper.	Mean	1.7	4.1	6.4	2.9
	Stand. Dev.	1.89	3.75	3.15	1.19
	No. of exper.	13	21	65	9
pH	Range	4.35-8.60	4.76-8.87	7.53-9.22	7.28-8.47
	Mean	7.69	8.03	8.51	8.10
	Stand. Dev.	1.03	0.57	0.44	0.27
	No. of specimens	42	105	108	27
Consistency	No. (%)				
	of specimens				
	Viscous	29 (64.4%)	64 (59.8%)	92 (64.8%)	27 (96.4%)
	Fluid	14 (31.1%)	30 (28.0%)	32 (22.5%)	1 (3.6%)
	Mixed	2 (4.4%)	13 (12.1%)	18 (12.7%)	0 (0.0%)
	Total	45	107	142	28
Opacity	No. (%)				
	of specimens				
	Opaque	26 (59.1%)	54 (56.3%)	104 (81.9%)	26 (96.3%)
	*Non-opaque	18 (40.9%)	42 (43.7%)	23 (18.1%)	1 (3.7%)
	Total	44	96	127	27
Columnar Cells	No. (%)				
	of specimens				
	Many	28 (68.3%)	76 (72.4%)	35 (83.3%)	19 (95.0%)
	†Few	13 (31.7%)	29 (27.6%)	7 (16.7%)	1 (5.0%)
	Total	41	105	42	20

* Non-opaque = transparent or translucent.

† Few cells = none or a small number of cells per field.

to that secreted spontaneously in the same period of time.

The one disadvantage in the use of clove oil as a standard stimulus arises from its being a mixture of several chemical compounds in variable proportions, rather than a single chemical individual. However, the chief component of this essential oil is eugenol (4-allyl-2-methoxy phenol) which comprises 82-87% of the mixture. Hence, it seemed that this compound may be responsible for a major part of the mucus-stimulating action of the clove oil, and, therefore, better suited for use as a standard mucus stimulus. We have investigated the secretory response of the gastric mucosa to topical application of aqueous emulsions of pure eugenol, and the results are presented in this report.

Procedure. The experimental technique was the same as that used previously for studying the action of clove oil and the other stimuli.³ The eugenol emulsions were prepared in concentrations ranging from $\frac{1}{4}$ to 5%. Acacia (5%) was used as an emulsion stabilizer in

the earlier experiments; later this was replaced by Tergitol Penetrant-4 (1/40%) with equally good results.[§] Control experiments with these substances alone showed that they exert no mucus-stimulating action at these concentrations; the fluid obtained after their application was the same as spontaneously secreted mucus in appearance and rate of formation. As in our previous work, the eugenol stimulus was administered only after the pH of the control (pre-stimulation) specimens had risen above 6.0. In general, collection of the stimulated specimens was continued until the rate of secretion fell to its control value. The pH's were determined electrometrically, with a glass electrode, and were reproducible to ± 0.02 of a unit. A total of 99 experiments (*i.e.*, stimulations) was performed with eugenol emulsions, using 9 dogs with Heidenhain pouches.

[§] Tergitol Penetrants were kindly supplied by the Carbide and Carbon Chemicals Corp., New York City.

undifferentiated sarcoma, one osteogenic sarcoma and one plasmocytoma. Metastases occurred in 3 instances. Among the tumors derived by transplantation there were 63 fibrosarcomas, and a few other miscellaneous growths. Variation in differentiation and growth rate was noted in different generations.

Discussion. We have arrived at no conclusion as to the nature of the carcinogenic agent in these experiments. Attention is called to the fact that the procedure described is one of the simplest known for the production of sarcomas. In this connection the production of rat sarcomas by Turner³ by embedding disks of bakelite (phenol-formaldehyde) is of interest.

In the course of certain surgical procedures, cellophane has been used as a covering or sheath,⁴ and left in the human body. We are not acquainted with any reports of the devel-

opment of sarcoma in man subsequent to its use, but this possibility should not be forgotten.

Conclusions. 1. Sarcomas were induced in albino rats by the insertion of regenerated cellulose film either subcutaneously or by wrapping it around one kidney.

2. These tumors occurred in about 35% of the rats surviving the operation more than 11 months.

3. The tumors were transplantable.

4. This is a simple method for inducing sarcomas experimentally.

5. The foregoing results in rats should be taken into consideration in the surgical use of cellophane in man.

³ Turner, F. C., *J. Nat. Cancer Inst.*, 1941, **2**, 81.

⁴ Ingraham, F. D., Alexander, E., Jr., and Matson, D. D., *New Eng. J. Med.*, 1947, **236**, 403.

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Eugenol as a Stimulus for Gastric Mucous Secretion*††

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In the course of investigations in this laboratory on the characteristics of gastric mucus, various types of stimulation have been studied. These included subcutaneous injection of pilocarpine, gentle mucosal massage, and topical application of a number of aqueous solutions and emulsions including ether (saturated), ethyl alcohol (50%), isotonic NaCl (0.17 N), hypertonic NaCl (0.5 N), clove oil (5%), mustard oil (1%), and

distilled water.^{1,2,3} Among the several objectives of these investigations was the discovery of a mucus-stimulating agent which induces no parietal secretion and therefore might be adopted as a standard stimulus for further work on the physiology of mucus secretion. Of the nine stimuli already studied, the 5% clove oil-water emulsion was clearly the most suitable for the purpose, as evidenced by the following characteristics of specimens obtained after a single application of this agent: (a) high viscosity, (b) high pH,⁴ and (c) a large total volume relative

* A preliminary report of this work was transmitted to the XVII International Physiological Congress (Hollander, F., and Lauber, F. U., *Communications XVII Internat. Physiol. Cong.*, 1947, p. 155).

† This investigation was conducted with the aid of grants from the Altman Foundation, and Wyeth, Inc.

†† The authors wish to express their thanks to Dr. Sophya Lazard for conducting some of the experiments.

¹ Hollander, F., Lauber, F. U., and Stein, J. J., *Am. J. Physiol.*, 1947, **149**, 724.

² Hollander, F., and Stein, J. J., *Am. J. Physiol.*, 1943, **140**, 136.

³ Hollander, F., Stein, J. J., and Lauber, F. U., *Gastroenterology*, 1946, **6**, 576.

⁴ Hollander, F., *J. Nat. Cancer Inst.*, 1945, **5**, 367.

eugenol, and eugenol acetyl salicylate,⁶ and it is possible that some of these substances are particularly potent as desquamating agents, even in small quantities.

Summary. The characteristics of gastric mucous secretion, stimulated by topical application of aqueous eugenol emulsion in several concentrations, have been investigated on 9 Heidenhain pouch dogs. A 5% emulsion of clove oil, which had previously been found to be superior to all other mucus stimuli, was used as a basis of reference for determining the secretory value of eugenol. It was found that 5% eugenol yields larger volumes of secretion, with a higher pH, than the clove oil. The percentages of specimens possessing high viscosity, opacity, and columnar cell content are lower for the eugenol than for the clove oil. Since we have already cited reasons³ for believing that *pure* gastric mucus is trans-

parent, cell-free, and of variable consistency, it may be that the secretion yielded by eugenol differs less from pure mucus than does the fluid obtained with clove oil.

Five per cent eugenol emulsion is the most effective stimulus to mucus secretion which we have found to date—especially since it yields larger volumes of mucus which tend to have a higher pH than those induced by any of the other stimuli. The latter characteristic indicates also that eugenol has virtually no stimulating effect on the parietal cells. Furthermore, this substance is a pure compound, the major component of clove oil, whereas the latter is a mixture of at least 7 different chemical substances. Since the minor components of the essential oil may also exert some physiological effect on the mucosa, their absence from the pure eugenol enhances its value as a standard. Hence, we propose to adopt an aqueous emulsion of eugenol as a standard stimulating agent for further work on the physiology of gastric mucous secretion.

⁶ Wood, H. C., Jr., and Osol, A., *The Dispensary of the United States of America*, 23rd ed., Philadelphia, 1943.

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The Influence of Coramine on the Liver of the Young Rat.

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Coramine (nikethamide) is a compound which provokes a number of different pharmacological actions. It is capable of curing black tongue in dogs by virtue of its close structural relationship to nicotinamide;¹ it is often employed as a medullary stimulant; and it has a pronounced ability to increase the liver weight of young rats.² Coulson and Brazda^{2,3} have presented evidence which in-

dicates that the unsubstituted nitrogen of the heterocyclic ring and the di-ethyl substitution of the amido nitrogen are both in part responsible for the liver enlargement which follows the administration of the compound.

In preliminary experiments the absolute liver weight increase caused by coramine by the end of a 28-day period could not be prevented by the inclusion of 1.2% methionine or 0.5% choline in the diet.² This weight increase suggested that the liver cells were being injured by the coramine and that this was followed by the very rapid regeneration which is seen in many types of liver injury. It was deemed desirable to design experiments

¹ Smith, D. T., Margolis, G., and Margolis, L. H., *J. Pharm.*, 1940, **68**, 458.

² Coulson, R. A., and Brazda, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 1.

³ Brazda, F. G., and Coulson, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 19.

Results. The data on characteristics of mucus specimens obtained after eugenol and 5% clove oil emulsions are presented in Table I. Because of the small frequencies in some of the groups of data, the values for $\frac{1}{4}$ and $\frac{1}{2}$ % eugenol were combined into a single group designated "low concentration," and those for 1 and 2% into an "intermediate concentration" group. The resulting increase in the number of specimens in each group augments the chance of obtaining a significant statistical evaluation of the observations. The frequency of the 5% eugenol data being large, these were retained alone as the "high concentration" group.

Total volume of mucus secreted in response to a single application of stimulus. The mean volume of secretion per experiment increases with the concentration of eugenol in the stimulus, from 1.7 ml for the "low concentration" group to 6.4 ml for the "high concentration." This latter value, for the 5% eugenol concentration, is significantly greater^{||} than that for 5% clove oil (2.9 ml per experiment). The datum for 1-2% eugenol ("intermediate" group) is also greater than that for 5% clove oil but the difference is not significant.

pH. As shown in Table I, the mean pH of eugenol-stimulated specimens increases with increasing concentration of the stimulus, the differences between the several groups being significant at or below the 1% level of probability.[¶] Corresponding to this, there is an elevation of both the upper and lower limits of the range, but the data become increasingly homogeneous, as evidenced by a decrease in the range itself and in the standard deviation. The mean pH for 5% clove oil is significantly lower^{**} than that of eugenol of the same concentration but is essentially the same as that for 1-2% eugenol. Since the 5% eugenol emulsion gives a higher mean pH and a lower standard deviation than any of the other

stimuli which we have previously studied,⁵ it is the most effective agent encountered to date for evoking an alkaline response from the stomach.

Consistency, opacity, and columnar cell content. Qualitatively, these physical characteristics of mucus were essentially the same for eugenol as for all the other topical stimuli, except mustard oil emulsion. Some of the specimens were distinctly fluid; others jelly-like or of intermediate viscosity. Some were transparent or translucent; others opaque. Some were cell-free or contained only cellular detritus; others contained many columnar cells—singly, or in ranks and clumps. In microscopic appearance, after being stained with toluidine blue, the eugenol-mucus smears were indistinguishable from those of clove oil-mucus. Numerically, the percentage incidences for the three categories of consistency shown in Table I are essentially the same for all 3 concentrations of eugenol; clove oil, however, gives a markedly higher percentage of viscous specimens. For opacity, the percentages of opaque specimens in the low and intermediate concentration groups are almost identical; whereas the value for 5% eugenol is considerably higher than these, and that for clove oil even greater.^{††} The values for columnar cell content show this same trend, although the differences are not significant statistically by the χ^2 -test ($P > 1\%$).

The consistently higher position of clove oil over eugenol, in regard to all three of these physical characteristics, is probably the result of the action of one or more compounds in the essential oil other than eugenol. In addition to the latter, clove oil contains vanillin, methyl alcohol, furfural, caryophyllene, acetyl

⁵ Hollander, F., Lauber, F. U., and Stein, J. J., in preparation.

^{||} This difference is significant at the 1% level of probability; $t = 3.3$, $n = 72$, $P = 0.18\%$.

[¶] The difference between the mean-pH's for the low and intermediate concentrations gave the following statistics in the t -test: $t = 2.6$, $n = 145$, $P = 1\%$. For the intermediate and high concentration groups, $t = 6.9$, $n = 211$, $P < 0.01\%$.

^{**} $t = 4.8$, $n = 133$, $P < 0.01\%$.

^{††} These differences in relative frequencies are statistically significant at the 1% level of probability, as shown by the χ^2 -test. For the first of these—with categories of opaque and non-opaque specimens, and "high" and "intermediate" concentrations of eugenol— $\chi^2 = 17.4$, $n = 1$, $P < 0.01\%$. For the second—with categories of opaque and non-opaque specimens, and 5% clove oil and "intermediate" concentrations of eugenol— $\chi^2 = 14.8$, $n = 1$, $P = 0.01\%$.

TABLE II.
The Influence of the Ingestion of Coramine on the Liver Glycogen of Young Rats.

Group	No. rats	Liver wt as % body wt		% liver glycogen	
		Range	Avg	Range	Avg
Control	11	3.63-5.27	4.42 \pm 0.09	1.40-3.75	3.09 \pm 0.13
1% coramine	12	5.56-7.66	6.81 \pm 0.11	1.66-3.28	2.39 \pm 0.09

in amounts calculated to be far in excess of any normal requirements. Methionine in such a massive dose caused a considerable decrease in the growth rate and choline provoked a slight decrease (Table I). In each case the growth rate was in proportion to the food consumed. The comparatively high protein diet supplemented with methionine or choline was chosen in an attempt to provide dietary conditions such as to minimize any liver damage.

The increase in liver weight occurred in surprisingly short time and the livers remained larger than in the controls for the full 28-day period in all of the three experimental groups (Table I). There was a gradual decline in liver wt/body wt ratio in the last 2 weeks in all 3 groups. It is evident that neither choline nor methionine is capable of modifying the increase in the liver wt/body wt ratio caused by coramine. The slight differences noted in the three groups may be explained by the decreased fat content in the groups receiving choline or methionine.

Those rats which received coramine had livers which showed some elevation in the fat content as early as 5 days (Table I) and continued to show roughly a constant level of fat for the full 28-day period. Both choline and methionine reduced the fat content of the liver to a level significantly below that of the coramine rats but slightly above that of the controls. The solid content of the livers was the same in all 4 groups. This becomes evident if one subtracts the excess fat in the coramine series prior to calculation. The absolute increase in liver weight must be due to an increase in both solids and water in the same proportion as that found for the control series.

Since the increase in liver weight was not due to a disproportionate increase in water or fat, experiments were devised to determine

whether this might be due to glycogen. Eleven rats, 7 females and 4 males, were placed on the control diet. Twelve rats, 7 females and 5 males, were placed on the 1% coramine diet. At the end of 6 days both groups of rats were killed and the liver glycogen was determined by the method of Good, Kramer and Somogyi.⁵ It was decided that a longer period was unnecessary (Table I). The results appear in Table II.

It is evident that the glycogen content of the livers taken from the coramine treated rats, although somewhat lower, was not seriously different from that of the control rats. Glycogen storage was not responsible for the liver enlargement. The increase in liver weight in the glycogen series was less marked than in the series reported in Table I. This discrepancy may possibly be explained by the fact that this experiment was conducted during the hottest time of the year. The general phenomenon of significant liver enlargement, however, is still apparent.

Microscopic examination showed that the livers from rats receiving coramine had intracellular fat globules. Close correlation existed between the histological picture and the gravimetric analysis for fat. A prominent feature of the liver parenchyma was the large number of mitoses and binucleate cells. There was a slight increase of interlobular connective tissue in the livers of the experimental animals. Aside from the significant decrease in fat in the livers of rats receiving coramine plus choline or methionine no other effects due to these lipotropic agents were observed. Livers from rats after the 28-day experimental period were little different from those of the 5-day groups.

Discussion. In spite of the fact that cora-

⁵ Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

TABLE I.
Effect of the Oral Administration of Choline or Methionine to Young Rats on a 1% Coramine Diet.†

No. days	Rat final wt	Liver wt wet	Liver wt dry	Liver wt as % body wt	Liver fat, % wet wt	Liver fat, % dry wt	Liver, % solids	Gain in wt, g/day
Control.								
5	63.9	3.05	0.97	4.80 ± 0.10*	4.83 ± 0.21*	15.11	32.23	+3.47
9	70.0	3.36	1.06	4.78 ± 0.09	4.22 ± 0.12	13.38	31.47	+3.30
14	99.7	4.78	1.54	4.79 ± 0.09	4.01 ± 0.27	12.41	32.10	+3.93
19	122.4	5.59	1.74	4.56 ± 0.10	3.52 ± 0.21	11.19	31.46	+4.03
24	132.4	6.10	1.96	4.60 ± 0.08	4.09 ± 0.14	12.94	31.93	+3.56
28	152.9	6.56	2.08	4.27 ± 0.09	4.27 ± 0.07	13.47	31.69	+3.85
1% Coramine.								
5	52.2	4.32	1.49	8.31 ± 0.12	6.72 ± 0.54	19.82	34.14	+1.50
9	66.4	5.81	1.89	8.76 ± 0.19	7.66 ± 0.42	23.33	32.53	+2.56
14	83.8	6.87	2.38	8.25 ± 0.18	8.62 ± 0.50	25.19	34.33	+2.76
19	97.6	7.04	2.44	7.31 ± 0.25	7.88 ± 0.39	22.55	34.69	+2.71
24	106.4	7.85	2.70	7.38 ± 0.14	8.22 ± 0.71	23.29	34.58	+2.70
28	119.3	8.35	2.93	6.98 ± 0.17	8.60 ± 0.19	24.17	35.17	+2.73
1% Coramine + 2.4% Methionine.								
5	39.1	3.09	0.94	7.94 ± 0.27	4.59 ± 0.19	15.06	30.45	-0.40
9	47.8	3.81	1.21	7.95 ± 0.14	5.19 ± 0.16	16.32	31.75	+0.26
14	63.1	5.32	1.80	8.33 ± 0.28	6.89 ± 0.87	20.89	33.48	+1.13
19	72.0	5.84	1.84	7.92 ± 0.17	5.40 ± 0.31	16.63	32.41	+1.41
24	84.7	6.22	2.04	7.44 ± 0.19	6.26 ± 0.52	18.88	32.45	+1.62
28	80.3	5.79	1.90	7.21 ± 0.10	5.49 ± 0.34	16.51	32.94	+1.34
1% Coramine + 0.5% Choline.								
5	40.1	3.37	1.04	8.52 ± 0.21	3.98 ± 0.18	12.85	30.72	+0.76
9	53.9	4.06	1.36	7.59 ± 0.21	6.68 ± 0.42	19.76	33.16	+1.95
14	75.3	5.53	1.81	7.37 ± 0.24	6.17 ± 0.65	18.49	32.40	+2.53
19	82.9	5.92	1.85	7.16 ± 0.16	4.74 ± 0.18	15.33	30.89	+2.39
24	95.2	6.26	1.98	6.55 ± 0.19	4.58 ± 0.23	14.27	31.62	+2.42
28	109.0	7.32	2.41	6.66 ± 0.13	4.52 ± 0.23	13.66	32.84	+2.55

$$* P.E. = \pm 0.6745 \sqrt{\frac{\sum(y)^2}{n(n-1)}}$$

† Each figure in the table represents the average value obtained from 5 males and 5 females.

which would give some information on the nature of the increase in liver weight and which would help to explain the action of the compound.

Experimental. To determine the rate of the liver weight increase 4 groups containing 60 rats each were placed on a diet which consisted of 25% casein, 55% starch, 15% cottonseed oil and 5% salts.⁴ Each rat also received a daily dose of one ml of Brewer's yeast extract (equivalent to one g of dried yeast) and one drop of Percomorph oil a week. The rats, weighing 35-45 g, were an inbred mixture of the Sprague-Dawley and Illinois strains. Coramine* was mixed into the diet at a level of 1% for all groups except the controls. One group received an additional

supplement of methionine at a level of 2.4% of the diet and one the equivalent of a 0.5% choline supplement in the form of choline chloride. At intervals of 5, 9, 14, 19, 24, and 28 days, 5 male and 5 female rats, each from a different litter, were killed out of each of the 4 groups. The livers were weighed, the fat and water contents were determined by analysis and a specimen was saved for the preparation of histological sections. These were stained by the routine hematoxylin-eosin method. The water content was determined by drying the livers to constant weight in a vacuum desiccator over concentrated sulfuric acid at about 30 mm Hg pressure. The dried livers were then used for the determination of the fat (total lipid) content. They were extracted twice with hot alcohol, four times with an alcohol-ether mixture and ten times with ether. The solvents were evaporated and the fat was determined gravimetrically.

Both choline and methionine were added

⁴ Hawk, P. B., and Oser, B. L., *Science*, 1931, 74, 369.

* The commercial product in 25% solution was used.

within 24 hours, even in the presence of large amounts of dialysed embryo extract containing embryonin as a growth promoting factor.⁵ Evidently some low molecular weight substances essential for the metabolism of tissue cells have been removed by the dialysis. The action of these diffusible substances differs from the action of embryonin, for the diffusible factors are able to maintain the cells in good condition, though in the absence of embryonin no growth is obtained. Embryonin, on the other hand, acts only in the presence of the diffusible substances; it does not maintain the cells in dialysed media. For this reason, we call embryonin a growth promoting factor, while for the diffusible substances of low molecular weight we use the term accessory growth factors. These two terms are used throughout in order to distinguish between the two types of substances and between their different modes of action.

Preparations of accessory growth factors that induce the growth of tissue cells placed in dialysed media (containing embryonin) have been obtained from blood, serum, kidney, heart, yeast, and barley malt.⁶ The partial purification of malt extracts⁷ showed that it is possible to remove considerable quantities of inactive material from these mixtures without any significant interference with the biological properties of the extract, which suggests that only a few of the substances present may be responsible for its action on tissue cells. Recently, we were able, by a treatment with living bakers' yeast, to remove from such an extract all the fermentable carbohydrates. With this product, we were able to show the importance of glucose, mannose, and fructose in cell metabolism.⁸ The further purification was only partially successful, but from the diffusible substances present in calf embryo

muscle we obtained very potent preparations.⁹

Accessory Growth Substances in Dialysates from Embryo Juice. In these experiments, the test material consisted of chicken heart fibroblasts cultivated for 5-8 passages in the usual manner before being placed in the dialysed media in Carrel flasks. The technic has been described previously.^{5,6,7} The crude material is prepared as follows:

The muscles are removed from 5 calf embryos, passed through a meat chopper, and an equal volume of Ringer's solution is added. After standing for about one hour at room temperature, with occasional stirring, the mixture is pressed through gauze. The resulting extract (8 liters), corresponding to 4 liters of muscle, is dialysed in cellophane tubings against 2 portions of 8 liters each of distilled water for 2 days. Toluene or xylene must be added in sufficient quantities as preservatives, otherwise inactive products are obtained. The combined dialysates containing about 75% of the dialysable substances of the extract are evaporated almost to dryness *in vacuo* in a water bath at 40-50°. The residue is treated with 120-150 ml of a mixture of 1 vol. glacial acetic acid and 2 vol. of methanol (possibly with slight heating) and, as a result, most of the inorganic salts (chlorides) are left undissolved, while all the active substance is found in the solution. After centrifugation, it is precipitated with 3 vol. of absolute ethyl alcohol, and the precipitate is isolated by centrifugation or filtration on a sintered glass filter and treated with absolute alcohol and dry ether. The product is very hygroscopic and must be treated with utmost care at this point. It is immediately dried *in vacuo* over H₂SO₄ and solid NaOH in a desiccator. When dry, it can be kept indefinitely in a stoppered bottle. Preparation V-521 yielded 32.4 g, corresponding to 10.8 mg substance per ml of embryo muscle. It contained 3.44% N, 1.37% inorganic P, and 1.41% organic P. Addition of 0.2 ml of a solution containing 10-33 mg of this product per ml (in physiological saline, neutralized and sterile-filtered) to a Carrel flask containing dialysed media restores the ability of the media to promote the normal appearance and growth of the tissue cells.

⁵ Fischer, A., *Acta Physiol. Scand.*, 1941, **2**, 143.

⁶ Fischer, A., and Astrup, T., *Plügers Arch.*, 1942, **245**, 633; Astrup, T., Fischer, A., and Volkert, M., *Acta Physiol. Scand.*, 1945, **9**, 134.

⁷ Astrup, T., and Fischer, A., *Acta Physiol. Scand.*, 1945, **9**, 183; 1946, **11**, 187.

⁸ Astrup, T., Fischer, A., and Oehlenschläger, V., *Acta Physiol. Scand.*, 1947, **13**, 267.

⁹ Astrup, T., Fischer, A., Ehrensward, G., and Oehlenschläger, V., *Acta Physiol. Scand.*, in press.

mine seems to promote a very rapid "regeneration" of liver tissue, the liver shows relatively little damage even after 28 days on a coramine diet. It is quite possible that necrosis or cirrhosis or both might have been produced by continuing the experiment for several months. Blumberg and Grady⁶ found that over 300 days were required to produce fibrosis in rats with fatty livers.

It is surprising that the full effects of coramine on the liver wt/body wt ratio are manifest in 5 days or less and that no obvious progressive change occurs from then up to the 28th day. The growth rate was depressed, but not seriously, and all of the 180 experimental animals appeared to be in good condition at the time of autopsy.

Essentially none of the results presented, either chemical or histological, indicate any serious abnormality in the liver parenchyma of the coramine treated rats. A hypothesis as to the nature of the action of coramine on the liver cannot, however, be advanced. The rapid-

⁶ Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

ity and degree of enlargement of the liver superficially resembles the effect produced in organs by their respective trophic hormones. Whether this resemblance is more than superficial remains to be determined.

Summary. (1) Coramine produces a great increase in the liver wt/body wt ratio in growing rats. (2) This increase which is manifest within 5 days or less and which is still apparent for at least 28 days cannot be prevented by the ingestion of choline or methionine. (3) The percentage of water and glycogen in these livers is essentially the same as in the controls. Coramine causes some elevation in the fat content which may be prevented by the administration of choline or methionine. (4) On microscopic examination, aside from the fat globules observed in the livers of rats receiving coramine alone, no marked pathological changes were evident. Binucleate cells and mitoses were present in large numbers.

The authors wish to express sincere thanks to Dr. T. Hernandez for carrying out the microscopic examination of the histological sections.

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Growth of Animal Tissue Cells in Artificial Media.*

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Multiplication and growth of tissue cells *in vitro* depend on the presence of two kinds of substances in the culture media. Substances of a protein nature present in extracts from embryonic tissues were shown by Carrel¹ to act as growth promoting factors. These high molecular weight substances are present only to a very limited extent in blood plasma or serum. Attempts at the isolation of these very labile substances, grouped by us under

the name "embryonin";² have shown that they can be purified by the extraction methods outlined by Hammarsten³ for the isolation of a nucleo-protein. Other methods of purification have so far failed. These results have been confirmed by Davidson and Waymouth.⁴

When the culture media (plasma, serum, embryo extract) are dialysed against Ringer-glucose solution, and Tyrode's solution is added (furnishing inorganic phosphate and bicarbonate), the tissue cells disintegrate

* Aided by grants from Rask-Orsted's Fond and from Kong Christian den Tiendes Fond.

¹ Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

² Fischer, A., and Astrup, T., *Pflügers Arch.*, 1943, **217**, 34.

³ Hammarsten, E., *Z. physiol. Chem.*, 1920, **100**, 141.

⁴ Davidson, J. N., and Waymouth, Ch., *Biochem. J.*, 1945, **30**, 188.

TABLE I.
Mixture V-605 of Biologically Active Substances Tested on Tissue Cultures.
(Mg of substances contained in 1 liter solution.)

NaCl	7500	<i>l</i> (—)-Lysine, 2HCl	15
KCl	200	<i>l</i> (—)-Histidine, HCl	5
CaCl ₂	200	<i>l</i> (—)-Arginine	2
MgCl ₂	100	<i>d,l</i> -Valine	14
Na ₂ HPO ₄	50	<i>l</i> (—)-Leucine	9
NaHCO ₃	1000	<i>d,l</i> -Isoleucine	10
		<i>d,l</i> -Threonine	12
FeCl ₂	0.6	<i>d,l</i> -Phenylalanine	7
CuCl ₂	0.2	<i>l</i> (—)-Tryptophan	2
MnCl ₂	0.3		
ZnCl ₂	1.0	<i>d,l</i> -Methionine	6
CoCl ₂	0.01	Choline (as hydrochloride)	10
		Creatine	10
Glucose	800	Nicotinic acid	0.3
Mannose	100		
Galactose	100	Cystine	5
Inositol	20	Glutathione	5
Adenosinetriphosphate	200	Pantothenic acid	0.07
Fructose-diphosphate	100	Biotin	0.007
β -Glycerophosphate	100		
Inosinic acid	30	<i>p</i> -Aminobenzoic acid	1
		Hypoxanthine	100
Cozymase	5		
Thiamine	3	Sodium succinate	10
Riboflavin	0.2	" fumarate	10
Pyridoxine	0.3	" malate	10
		" oxaloacetate	10
Glutamine	250		
		Ascorbic acid	2
		Methylnaphthohydroquinone-sulphate	0.005

Among the substances of high molecular weight are the growth-promoting substances of protein nature contained in the embryo tissue juice, the presence of which are necessary for cell multiplication. Cell survival, however, is possible without this addition. Further, the proteins of serum may play a role that has not yet been clarified. And then there are the substances of low molecular weight contained both in the embryo tissue juice and in the serum.

By dialysing the media, as we do, the problems are simplified, for we retain all the high molecular weight substances and have only to substitute the low molecular weight, dialysable substances by a synthetic mixture. We feel that it is necessary to distinguish clearly between the effects of the dialysable and of the non-dialysable substances and to solve these two questions separately. Further, we always add to our media a surplus of dialysed embryo tissue juice in order to furnish the growth-promoting factor and thus to obtain active growth of the cells. In this

manner, we have a much more rigid proof of the ability of the added mixture to provide accessory growth substances than is the case when only the life of the cells is maintained. In this respect, our investigations differ from those of most previous authors.

We next proceeded to devise a mixture of all the substances of biological importance known to be present in animal tissue, and undertook to use them in concentrations comparable with those found in the living organism. In this manner, we devised Mixture V-605, described in Table I. The mixture was made from neutralized concentrated sterile solutions of the various groups of components, and 1 ml was used in the medium in a Carrel flask. Tested in the manner described, very intense growth and normal appearance of the cells were obtained even for cultures of small size. In the absence of glutamine, the mixture was rather active when tested on large cultures, but almost inactive on small cultures. Glutamine alone was quite inactive. Removal of the group containing the organic phos-

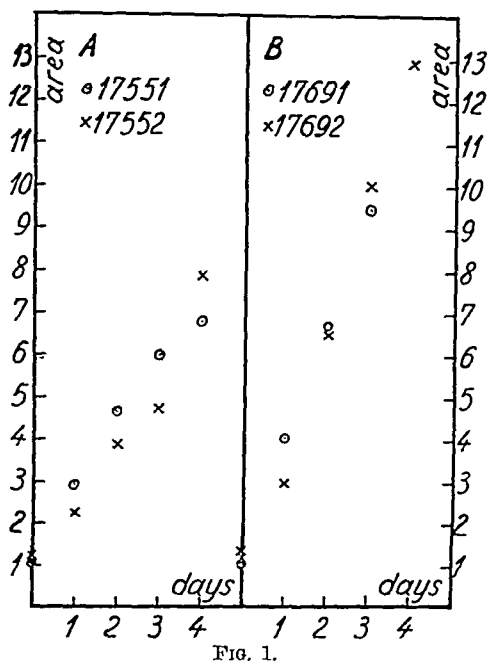


FIG. 1.

A. Effect on tissue cells of soluble calcium salts prepared from calf embryo dialysate after removal of inorganic phosphates. Culture No. 17551: Dry preparation isolated from dialysate (V-521-1). Culture No. 17552: Soluble Ca-salts (V-521-61). (Abseissa: Days. Ordinate: Relative growth area.)

B. Effect on tissue cells of a calf embryo dialysate after treatment with yeast and removal of inorganic P. Culture No. 17691: Soluble Ca-salts before yeast treatment (V-521-61). Culture No. 17692: Soluble Ca-salts after yeast treatment (V-521-74).

With barium acetate it is possible to precipitate all the active substances together with all the phosphorus. After dissolving this precipitate and removing the Ba^{++} , all inorganic P may be precipitated with Ca^{++} without significantly decreasing the activity.

A dry preparation, V-537-1, prepared as described, contained 0.227 mg N, 0.1290 mg inorganic P, 0.0406 mg organic P, and 2.50 mg sugar per ml solution.

1.48 g of V-537-1 (corresponding to 200 ml original solution) are dissolved in 20 ml water, and 20 ml ethanol is added. After making the solution just alkaline to phenolphthalein with 2N NaOH, 2 ml 30% Ba-acetate solution are added. The precipitate is removed by centrifugation, dissolved in water by means of diluted HCl, Ba^{++} is removed with sodium sulphate, the centrifu-

gate diluted to 200 ml, made alkaline to phenolphthalein with 2N NaOH, and 4 ml 10% $CaCl_2$ solution, saturated with $Ca(OH)_2$, is added. The solution is neutralized, concentrated *in vacuo* to 50 ml (4 times the original concentration), sterile-filtered and tested as usual. The solution is almost as active as the original product, and contains, per ml of the original concentration, 0.053 mg N, no inorg. P, 0.0246 mg org. P, and 0.56 mg sugar (preparation V-537-11).

The mother liquor from the Ba-acetate precipitation and the calcium precipitate were both almost inactive (Fig. 1 A).

By treatment with yeast, it is possible to remove part of the sugar in the preparations, and, at the same time, the organic phosphates are almost completely transformed into inorganic phosphates and may be removed as such by treatment with $CaCl_2$. These operations do not interfere significantly with the biological action of the preparations. Thus, preparation V-521-74 contained originally, per ml, 3.70 mg sugar, 0.190 mg inorg. P, and 0.161 mg org. P. After treatment of 200 ml with 10 g of bakers' yeast, overnight at room temperature, and removal of inorg. P with $CaCl_2$ -solution at pH 8-9, there remained 1.34 mg sugar per ml but no inorg. or org. P. Fig. 1 B shows the activity tested in 4 times the original concentration.

Synthetic Accessory Growth Substances. The first attempt of any significance to devise a synthetic medium was made by Baker and Ebeling.¹⁰ But because their results were based on the use of a digest of whole blood, they are of little use in disclosing the mechanism of cell nutrition, even though a medium made in this manner may be useful for other purposes.

The inability of embryo juice alone to furnish the substances necessary for normal growth of tissue cells was clearly demonstrated by Baker¹¹ who showed that serum is needed to furnish additional nutriment. Work on a synthetic medium must proceed, therefore, along 2 lines, for substances of both high and low molecular weight are involved.

¹⁰ Baker, L. E., and Ebeling, A. H., *J. Exp. Med.*, 1939, **69**, 365.

¹¹ Baker, L. E., *J. Exp. Med.*, 1939, **69**, 625.

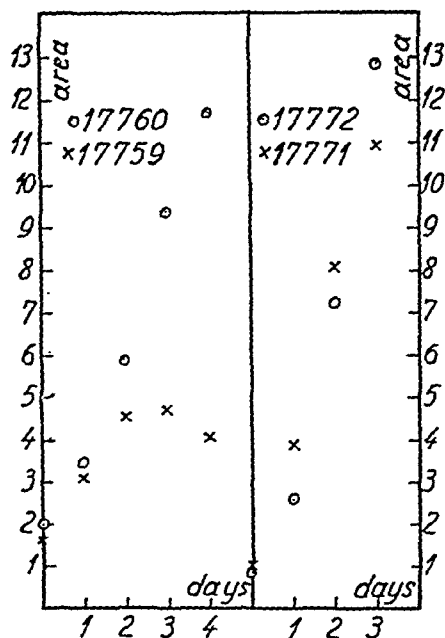


FIG. 3.

Effect of synthetic mixtures V-612 and V-614 on tissue cells. Culture No. 17759: Mixture V-612 without glutamine. Culture No. 17760: Mixture V-612. Culture No. 17771: Mixture V-612. Culture No. 17772: Mixture V-614.

cycle). It was surprising to us that the tissue cells would show normal growth in the absence of several of these substances. It may be that during the relatively short growth period (5-8 days) the constituents of the original tissue fragment suffice for the metabolism of the tissue cells, or that some of the components otherwise considered to be low molecular weight substances may be retained in the dialyzed plasma in combination with proteins. It is known, for example, that the heavy metal ions and certain organic components, *c. g.*, vitamin B₆, may be separated from proteins only with difficulty.

The vital importance of glutamine was expected in view of the relation between its function as a growth factor for certain streptococci and its role in streptococcal glycolysis, as found by McIlwain.¹² The importance of fructose-diphosphate is well understood in relation to its action in fermentation, and it may furnish a sufficient amount of hexose-diphosphate for the glycolytic system during

the first hours after transplantation of the tissue fragment and before the cells themselves are able to regain all their normal enzymatic functions. It is important in this connection, however, to remember that it seems possible to remove all organic phosphorus from the previously described accessory growth substances contained in dialysates from embryo tissue juice without interfering with the biological activity. These products contain no glutamine and their mode of action may be different from that of the synthetic mixture described here.

Recently, White¹³ has attempted to devise a completely synthetic medium. Our results are not in accordance with those of previous authors, and especially not with those of White, and though we consider it too early to discuss these differences a few points may be stressed. First of all we use a different technic, which in our opinion simplifies the problems in question by making possible a stepwise solution of the very complicated phenomenon of tissue growth. It is of special importance that while previous authors have preferred to work with media intended for maintenance of the cells for long periods, we use the active growth of cultures over short periods as a measure of the ability of the media to furnish the necessary substances. In this respect, it is of interest to note that two of our most important substances, *i. e.*, glutamine and fructose-diphosphate, are absent from the mixture described by White. Also, though White found that fibroblast migration is very much more active during the first few days in dextrose than in sucrose, he considers it probable that sucrose is superior to dextrose if the nutrient is not to be renewed frequently. In our experience, the tissue cells are completely unable to metabolize sucrose (saccharose).⁶ We suppose, therefore, that the tissue cells in White's experiments use the carbohydrates (dextrose) contained in the tissue pulp used for cultivation, and that the cells of the pulp would survive equally well if no sucrose at all were added. White seeks to avoid unknown constituents in the culture media (*c. g.*, serum, peptone, fibrin-digest, tissue extract, and sim-

¹² McIlwain, H., *Biochem. J.*, 1946, 40, 67, 460.

¹³ White, P. R., *Growth*, 1946, 10, 231.

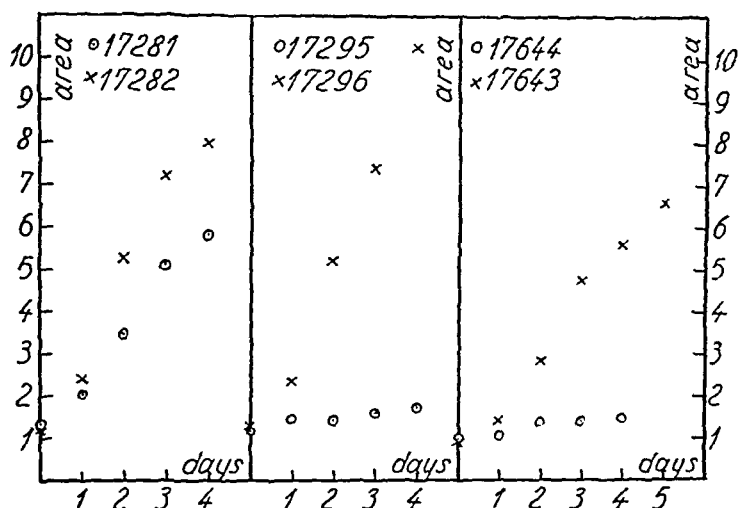


Fig. 2.

Effect of synthetic mixture V-605 on tissue cultures. Culture No. 17281: Mixture V-605 without glutamine. Culture No. 17282: Mixture V-605. Culture No. 17295: Glutamine alone. Culture No. 17296: Mixture V-605. Culture No. 17643: Mixture V-605. Culture No. 17644: Mixture V-605 without organic phosphates.

phates yielded a quite inactive mixture. Examples are shown in Fig. 2. Most of the other substances, however, could be discarded without influencing the action of the mixture on the cells, and in this manner we arrived at mixture V-612 (Table II), in which we have further increased the concentrations of organic constituents, except glutamine, with good results. Fig. 3 shows the action of this mixture with and without glutamine, tested on comparatively large cultures. The effect of glutamine is evident. The concentration of glutamine could be lowered to one-tenth without significantly interfering with its supplementary effect on the mixture. Further experiments show that it is possible also to discard β -glycerophosphate and inosinic acid, thus retaining only fructose-diphosphate (Fig. 3). Removal of the amino acids significantly decreases the activity. The importance of each individual amino acid has yet to be studied.

Discussion. A comparison of Tables I and II shows the substances not essential for growth of tissue cells under the conditions of the experiments. They include the heavy metals, known for their catalytic action on certain enzyme reactions, adenosine-triphosphate, cozymase, the different

TABLE II.
Simplified Mixtures V-612 and V-614 for Use with Tissue Cultures.
(Mg of substances contained in 1-liter solution.)
(β -glycerophosphate and inosinic acid were omitted in mixture V-614.)

	Mg
NaCl	7500
KCl	200
CaCl ₂	200
MgCl ₂	100
Na ₂ HPO ₄	50
NaHCO ₃	1000
Glucose	2000
Fructose-diphosphate	200
(β -Glycerophosphate	200)
(Inosinic acid	60)
<i>l</i> (-)-Lysine, 2HCl	30
<i>l</i> (-)-Histidine, HCl	10
<i>l</i> (-)-Arginine	4
<i>d,l</i> -Valine	28
<i>l</i> (-)-Leucine	18
<i>d,l</i> -Isoleucine	20
<i>d,l</i> -Threonine	24
<i>d,l</i> -Phenylalanine	14
<i>l</i> (-)-Tryptophan	4
Cystine	10
Glutathione	10
Glutamine	250

vitamins tested, choline, creatine, and the C₄-acids (functioning in the Krebs

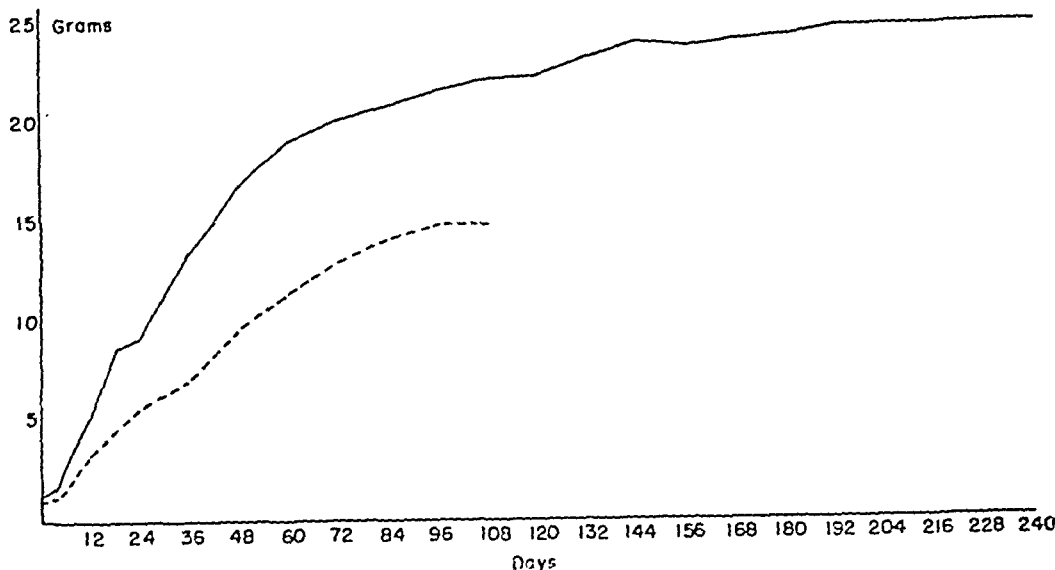


CHART 1.

This chart presents data on the growth rate of (a) normal male mice of the pBr subline of the NHO stock (solid line) and (b) hereditary dwarf mice of the same stock (short dash line). Time in days is given on the base line, body weight in grams is given on the vertical line.

teristics such as precocious opening of the vagina, delayed opening of the vagina even up to one year of age, and complete imperforate vagina, increased fecundity, precocious sexual maturity, increased fertility, excessively large first litters, rapid growth rate with the production of giant mice weighing up to 86 g of weight, and precocious and complete sterility. (5) Numerous cases of somatic mosaics especially involving hair pigmentation, and (6) the production of many germinal mutations involving hair color, eye color and distributional patterns of pigmentation.²⁻⁸

Recently Carr⁹ has reported 7 biological variants produced in mice by the subcutaneous injection of 1:2:5:6-dibenzanthracene. He reports 1. Hydrocephalus, 2. Absence of left

horn of the uterus, 3. Brain hernia, 4. Brown, 5. Pink eye, 6. Recessive spotting, and 7. Chin-chilla. The last 4 of these variants were proven to be genetic recessives.

It is a well recognized fact that the carcinogens are inhibitory in action.¹⁰ Thus when one injects a mouse with one of the carcinogens the normal growth rate is inhibited. In the pBr subline of the NHO descent whose ancestry has been injected with methylcholanthrene for many generations and the mice selected toward induced cancer resistance, the mice very seldom weigh more than 25 g when fully grown. The normal growth rate for mice of this subline is given on the solid line of Chart 1. On the 31st of March, 1947, it was noted that a pair of these pBr mice had produced a litter of young in which there were 4 normal and one dwarf mouse. These mice and their close relatives have produced, to date, a total of 121 mice in 21 litters in which, at least, one dwarf has appeared. Of these 121 mice, 92 were normal and 29 dwarfs. On a 3:1 basis one would expect 90.6 normals to 30.2 dwarfs. Thus it is obvious that dwarfism is a simple mendelian recessive.

² Strong, L. C., *Arch. Path.*, 1945, **39**, 232.

³ Strong, L. C., *J. Nat. Cancer Inst.*, 1945, **5**, 339.

⁴ Strong, L. C., *Nat. Acad. Sciences*, 1945, **31**, 290.

⁵ Strong, L. C., *Yale J. Biol. and Med.*, 1946, **18**, 145.

⁶ Strong, L. C., *Yale J. Biol. and Med.*, 1946, **18**, 359.

⁷ Strong, L. C., *Science*, 1946, **103**, 554.

⁸ Strong, L. C., *American Naturalist*, 1947, **81**, 50.

⁹ Carr, J. C., *Brit. J. Cancer*, 1947, **1**, 152.

¹⁰ Boyland, E., Edgar Allen Memorial Lecture, New Haven, Conn., Oct. 8, 1947.

ilar products), but in the pulp of macerated tissues used for cultivation he introduces large amounts of cell constituents of both high and low molecular weight. They may be removed by repeated washings either before or after they are placed in the culture tubes, and thus the difference between his results with dextrose and sucrose may be explained. The active growth of his cultures of tissue cells indicates the presence of the growth-promoting substance "embryonin" in his media, this substance being present in the macerated cells. A comparison with controls consisting of cultures made from washed tissue pulp would have been desirable.

Summary. 1. The difference between growth-promoting substances and accessory growth substances and between their different modes of action is pointed out. 2. From dialysates from embryo juice, preparations may be obtained which completely restore the ability of the dialysed culture media to induce normal growth of the tissue cultures. They may be prepared free from organic phosphates and glutamine. 3. A synthetic mixture is described which has the same properties of restoring the dialysed media. The most important of its components seem to be glutamine and fructose-diphosphate.

16199

Hereditary Dwarfism in the Descendants of Mice Receiving Methylcholanthrene—Parallel Induction.

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The following biological variants have been obtained in the descendants of mice whose ancestry have been injected with methylcholanthrene for many generations. (1) Several germinal mutations changing susceptibility to tumors induced at the site of the injection of the carcinogen. (2) Germinal mutations altering the incidence of specific types of tumors, following the injection of the methylcholanthrene and subsequently under spontaneous conditions in their untreated descendants—such as bronchiogenic carcinoma, leiomyo-sarcoma of the uterus, and gastric lesions of several histological types. (3) Many embryological disturbances such as dextrocardia, dextroversion, situs inversus, anovariae, absence of the cervix and vagina, absence of one kidney together with the absence of the horn of the uterus on the same side, craniorachichisis, several eye lesions, and

possibly (?) twins. (Twenty-two pairs of twins have been obtained in mice which have been injected with methylcholanthrene. In view of the reported rarity of this condition in mice (only three cases could be found in the literature) it would seem that twinning in these methylcholanthrene-treated mice is highly significant. However, in more recent work especially by W. F. Hollander,¹ it has been found that twinning or placental fusions in mice are of very frequent occurrence. It is doubtful, therefore, whether the injected methylcholanthrene had much influence on the development of this biological variant.) The above biological variants are considered to be induced by methylcholanthrene when they have fulfilled one of the following criteria: either (a) they have occurred only among the injected mice or their immediate descendants and never among a comparable group of controls, or (b) they have occurred among the methylcholanthrene-treated mice at an increased frequency over the controls. (4) Several physiological and morphological charac-

* This experiment has been made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund, and The American Cancer Society, Committee on Growth.

¹ Hollander, W. F., unpublished data.

induced in mice following the subcutaneous injection of methylcholanthrene for many generations is hereditary dwarfism. This condition is inherited as a recessive. The relation of this mutation to the general problem of parallel induction is briefly indicated. Thus it is becoming more obvious that both non-

genetic and genetic biological variants are being produced in mice by methylcholanthrene. The mechanism involved may be different for the different variants obtained but perhaps there may be a common denominator in them all.

16200

Effect of Muscular Fatigue on Histamine-Provoked Ulcer with Observations on Gastric Secretion.

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Complete physical rest is frequently mentioned by internists as a valuable adjunct in the medical management of peptic ulceration. This precept has been derived primarily from clinical observation as there are few if any experimental studies in recent medical literature which deal with the effects of muscular fatigue on alterations in gastrointestinal tract function particularly as they pertain to the ulcer diathesis.

It is the purpose of this report to indicate our observations on the effect of moderately severe muscular fatigue on the incidence of the histamine-provoked ulcer and upon gastric secretion in dogs.

Method of Study. Experiment 1. Fifteen healthy, dewormed dogs weighing from 26 to 54 lb, of young to medium age, were run from 3 to 4 hours consecutively each day in the morning on 5 to 6 successive days on an electrically driven endless belt treadmill. The dogs were allowed a 10-minute rest period each hour and water *ad lib*. The treadmill speed was calculated at 1.5 to 2.0 miles per hour which amounts to a brisk walking pace for the average sized dog. The slope of the treadmill was varied between an incline of 5 to 20° from horizontal depending on the

dogs' physical condition. Just prior to being placed on the treadmill each day, the dogs were given an injection intramuscularly of 30 mg of histamine base-in-beeswax mixture prepared after the method of Code and Varco.¹ The dogs were fed a diet of horsemeat, milk, dog biscuit each day after finishing their period on the treadmill. Rectal temperatures were taken before the start of the exercise and after each hour of exercise as a gross indication of the degree of muscular effort.

As a control series, 13 animals of comparable size, age, and health were fed and injected daily with histamine as above, but were not run on the treadmill. In addition 2 dogs were run on the treadmill as above, but were not given histamine. In these 2 animals the effect of the muscular exercise on hemoglobin, hematocrit, leukocytes differential count, and blood sugar was studied. All animals were sacrificed with examination of their gastro-intestinal tracts at the end of their test period of 5 to 6 days on the treadmill and/or 5 to 6 consecutive daily intramuscular histamine-in-wax injections.

Experiment II. Studies on gastric pouch secretion were made using 2 Heidenhain-type pouches made over one year prior to the onset of these experiments. Both animals used were

* National Cancer Trainee. This work is supported in part by the U. S. Public Health Service, the Augustus L. Searle Fund and the John and Mary R. Markle Fund for Surgical Research.

¹ Code, C. F., and Varco, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1940, 44, 475.

The growth rate for dwarf mice up to 108 days of age is given on the short dash line of Chart 1.

The dwarfs are smaller in size than the controls at birth and appear to be restless. They deviate strikingly from the controls at 2 to 3 days of age and, in competition with their normal siblings, they have invariably died before the weaning age of 30 days. A few days before death they lose weight progressively. However, when their normal siblings have been removed from the mother, the dwarfs will live for a longer time. So far only 5 have reached 100 days of age. Of these one pair has reached sexual maturity and has produced 16 young in 2 litters—all of which were dwarfs. One of these young was also a waltzer but unfortunately died before reaching sexual maturity, so that the biological nature of this waltzing could not be determined.

It is perhaps more than a coincidence that the mutations reported by Carr are the same as have been obtained in my laboratory. All 4 of his mutants have occurred several times in the descendants of mice which have been injected with methylcholanthrene. In addition, however, I have obtained many other germinal mutations, notably three types of dominant spotting, etc. One may entertain the possibility that perhaps there may be a specificity of action of the inducing system upon the genetic mechanism, an idea that has been mentioned previously. It must be borne in mind, however, that the first mutations that have been induced with methylcholanthrene and with 1:2:5:6-dibenzanthracene have been at loci which have given rise to spontaneous mutations in the past—thus indicating perhaps a greater mutability than other loci in the germ plasm of mice. The greatest difference between Carr's data and my own is, apparently, in the greater frequency of dominant mutations in my series. For example, with methylcholanthrene the recessive gene *b* has mutated to dominant *B* eight times, whereas the gene *B* has only mutated to *b* once. It is also clear that both non-genetic and genetic biological variants are being produced in separate laboratories. In mice, many biological variants occur for which no genetic evidence

of inheritance can be obtained. It is consequently better to use the term biological variant until evidence of inheritance can be established. When this evidence is forthcoming the variant thus becomes a mutant. Among the most interesting non-genetic variants are the somatic mosaics being induced with methylcholanthrene. The fact that both methylcholanthrene and 1:2:5:6-dibenzanthracene are powerful carcinogens and that consequently the experimental animals are giving rise to a multiplicity of cancers should not be lost sight of.

Parallel induction maintains that an induction system may influence both the soma and the germ plasm—perhaps conditioned by some biological bond between the gene and some characteristic of the soma conditioned by gene action. This appears to be the case in the induction of gastric lesions under the influence of methylcholanthrene. This gastric lesion became hereditarily established by a mutation on the brown tagged chromosome.⁵ The production of hereditary dwarfism in mice seems to be a second case of parallel induction. That is, that the induction system of methylcholanthrene or one of its metabolites has inhibited the normal growth rate of mice for several generations. When a germinal mutation took place it took place in the direction of the inductive influence. However, this has not always been the case. For example, the giant mice referred to previously apparently originated by some biological change (perhaps germinal) which was counter to the trend of biological induction.

The embryological disturbances may be brought about, perhaps, by an inhibitory action of the carcinogen at a critical period of the development of the embryo. For example, the absence of the cervix could be explained by the fact that perhaps the distal end of the Müllerian duct did not develop normally. The carcinogen is also inducing mutations and at the same time bringing about changes in the soma leading toward neoplasia. This multiplicity of biological effects by the injection of a pure compound may eventually be better understood when the metabolism of the carcinogen is more fully understood.

Summary. Among the germinal mutations

TABLE II.
Production of Ulcer in Normal Dogs.
(30 mg histamine-base in Beeswax daily.)
Control Series—No Muscular Fatigue.

Dog No.	Initial wt, lbs	No. of hist. inj.	Wt. loss lbs.	Results
960	40	6	4	Negative gastrointestinal tract
980	31	5	—	" " "
993	35	5	1	Single duodenal erosion
5	37	5	2½	Negative gastrointestinal tract
11	47	5	4	" " "
52	22	5	2½	" " "
65	32	5	4	Duodenal ulcer
380	30	6	4½	Negative gastrointestinal tract
282	23	5	3¾	" " "
870	17	6	0	" " "
996	15	5	0	" " "
12	18	5	2	Single duodenal erosion
51	21	5	1½	Negative gastrointestinal tract

No. of dogs injected 13

No. of dogs with ulcer and/or erosions 3

TABLE III.
Effect of Muscular Fatigue on Gastric Pouch Secretion.
Average Values.*

Dog No.		Fasting Vol., cc	Free acid	1st ½ hr after histamine†		2nd ½ hr after histamine		3rd ½ hr after histamine	
				Vol. cc	HCl output mg/½ hr	Vol. cc	HCl output mg/½ hr	Vol. cc	HCl output mg/½ hr
689	Resting	2.26	Absent 4 out of 9 trials	9.22	44.75	5.38	27.97	0.58	qns.
	Fatigue	0.21	Absent 7 out of 10 trials	3.79	16.90	4.13	20.31	0.35	qns.
21	Resting	0.37	Absent 1 out of 6 trials	8.20	37.26	15.43	89.11	3.31	18.55
	Fatigue	0.09	Absent 3 out of 5 trials	7.10	33.69	8.30	47.10	1.26	5.72

* Dog 689 tested 19 times.

Dog 21 tested 11 times.

† Dose of histamine phosphate 1.37 mg aqueous solution subcut.

which persists as long as 24 hours after the termination of a 4-hour period on the treadmill.

All dogs on the treadmill showed an elevation of rectal temperature which was roughly correlated with the amount of fatigue and their physical condition. The average elevation was 2°F.

Exp. II. *Results.* Studies of the effects of muscular fatigue on gastric secretion are summarized in Table III. Both dogs show a depression of volume and free acidity of the fasting (basal) pouch secretion. Moreover, the basal pouch secretion was noticeably more mucoid (often jelling in the bottom of the tube) when the animal was fatigued. Upon the background of an artificially introduced stimulus to gastric secretion in the form of

an injection of aqueous histamine the depressant effect of muscular fatigue becomes much more apparent upon the volume of gastric juice. After aqueous histamine stimulation in exercised animals there is also a consistent, but small, reduction in degrees of free and total acid in the pouch juice. However, the main depressant effect on the production of total hydrochloric acid in muscular fatigued animals is the reduction in volume of juice secreted. The results in Table III are reported in terms of weight or output of hydrochloric acid per unit time which is the most reliable criterion of the rate of acid secretion in the stomach. (Lim).²

Discussion. These results indicate that mod-

² Lim, R. K. S., *Am. J. Physiol.*, 1924, 69, 218.

TABLE I.
Production of Ulcer in Dogs Following Muscular Fatigue.
(30 mg histamine-base in Beeswax daily.)

Dog No.	Initial wt, lbs	Days on treadmill	Total hrs on treadmill	No. of hist. inj.	Wt loss lbs	Results
888	39	6	23	6	4	3 gastric ulcers
902	32	6	20.5	6	3	Negative G. I. T.
909	37	5	18	5	3	Duod. ulcer and erosions
916	34	5	18.5	5	2	Multiple duod. and jejunal erosions
948*	40	2	6	1	—	Duod. and jejunal erosions
951	37	5	19	6	3	Large duod. ulcer, antral erosions
956	26	5	19	6	7½	Large duod. ulcer, antral erosions
967	43	5	17	6	6½	2 duod. ulcers
979	45	5	20	5	4	Negative G. I. T.
994	40	5	19	5	2	Duod. erosions
7	32	3	12	3	5	Died, perforated duod. ulcer
16	43½	5	20	5	½	Negative G. I. T.
38	54	5	20	5	4	Duod. erosion
59	44	5	20	5	9	Multiple penetrat. duod. ulcers
375	38	5	20	5	5½	Negative G. I. T.

* Died in hyperthermia (rectal temperature above 108°F).

No. of dogs tested 15

No. of dogs with ulcer and/or erosion 11

in a good general state of nutrition, free of any infection and secreted clear pouch juice. To facilitate the collection of the gastric juice without spillage on the treadmill both pouches were made with a 30 French mushroom catheter as an outlet for the gastric juice. The test period was begun at 9:00 a. m. Routinely food pans were removed from cages at 5:00 p. m. on the day preceding the test insuring a 16-hour fasting period before each experiment. Water was allowed ad lib up to but not after the time of the onset of the experiment. The samples were collected at one-half-hour intervals. The pouches were tested alternately on and off the treadmill. Dogs tested on the treadmill were run for 20 to 30 minutes before the collection of gastric juice was begun. All samples were analyzed the same day for volume, free and total acid, and pH, volume permitting.

In order to test the depressant action of muscular fatigue on gastric secretion an artificially produced stimulation of pouch secretion was created using one-half milligram histamine-base (1.37 mg histamine phosphate) injected subcutaneously. All dogs were given one or more days rest between experiments. Diet consisted of horsemeat, milk, and dog biscuits fed at approximately the same time each day.

Exp. I. Results. The incidence of hista-

amine-in-beeswax provoked ulcer or erosion gastric and/or duodenal) following strenuous muscular exercise is shown in Table I. This incidence is to be compared with the results as shown in Table II of injection of the same dose of histamine-in-beeswax for a comparable length of time without the effect of fatigue. Of the 2 dogs which were run on the treadmill as outlined above but were not given histamine-in-beeswax injections neither developed ulcers or erosions. However, at autopsy one of these animals (No. 326) showed a remarkably severe duodenitis. The duodenum was fiery red in color due to submucosal hemorrhages from the pylorus to the point of attachment of the ligament of Treitz. Microscopically sections from this area showed intense dilatation of the capillaries within the mucosa with extravasation of red blood cells. The colon likewise showed a rather severe colitis, the mucosal surface being bright red in color due to congestion and submucosal hemorrhages. This appearance of the colon was quite a common finding at autopsy also in the dogs run on the treadmill and given histamine. (Table I).

The blood changes caused by this amount of exercise on the treadmill were a mild hemoconcentration, a moderate fall in blood sugar immediately following exercise, and a polymorphonuclear leucocytosis above 25,000

Attempts to Prevent Ergot Gangrene with Heparin and Dicumarol. Vascular Effects of Ergot by Fluorescein Technic.

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The suggestion that thrombosis, associated with ergot gangrene,¹⁻¹⁰ contributes to the vascular occlusion responsible for the resultant tissue necrosis,^{3,7} prompts the hypothesis that prevention of thrombosis should modify the gangrene. This hypothesis is supported by the observation that heparin prevents necrosis resulting from experimental "frostbite," a condition with which thrombosis is also associated.¹¹⁻¹³

The present study is concerned with the influence of heparin and dicumarol on the incidence of ergotamine induced gangrene in the rat.

Gangrene of the tail was produced in male and female albino rats (140-200 g) by the subcutaneous or intraperitoneal injection of a 1:1000 solution of ergotamine tartrate. Whenever a particular treatment was employed, litter mates served as controls. Preliminary studies revealed no influence of sex or route of administration of ergotamine on the incidence of gangrene.

Gangrene can be produced by a single injection of a suitable dose of ergotamine^{6,14-17} and such a procedure was employed. When doses of 3.3, 12.5, 25.0, 37.5 and 50.0 mg/kg were injected it was observed that the incidence of gangrene with the smallest dose was very low, while with the largest dose a high mortality occurred. The incidences of actual necrosis in 31, 17 and 13 normal rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 35.5, 43.3, and 15.4% respectively.

In the process of developing gangrene the appearance of the rats' tails passed progressively through several poorly defined stages. The earliest certain indication of impending necrosis was the appearance of a demarcating region of redness and tenderness, distal to which was an area of extreme pallor, with dark discoloration of the tip of the tail. This change was designated arbitrarily as the onset of gangrene, and on the basis of such criteria the time of onset of gangrene was determined in 115 rats (Fig. 1). A few animals began to develop gangrene within 24 hours of the injection of ergotamine. Approximately 50% of the animals which developed gangrene began to do so within 3 days and 100% within 7 days of the injection of ergotamine.

Rats given a single subcutaneous injection of 30 mg/kg of heparin in Pitkin's menstruum^{18,19} showed prolongation of the blood

¹ Kobert, R., *Arch. f. exp. Path. Pharmacol.*, 1883, **18**, 316.

² Dale, H. H., *J. Physiol.*, 1906, **34**, 11.

³ Lewis, T., *Clin. Sci.*, 1935, **2**, 43.

⁴ Kaunitz, J., *Arch. Surg.*, 1932, **25**, 1135.

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⁷ Yater, W. M., and Cahill, J. A., *J. A. M. A.*, 1936, **106**, 1625.

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⁹ Rubin, M. J., and Rapoport, M., *Arch. Int. Med.*, 1937, **59**, 714.

¹⁰ Thomas, R. M., *Yale J. Biol. and Med.*, 1940, **12**, 415.

¹¹ Lange, K., and Boyd, L. J., *Surg. Gyn. and Obst.*, 1945, **80**, 346.

¹² Lange, K., and Loewe, L., *Surg. Gyn. and Obst.*, 1946, **82**, 256.

¹³ Friedman, N. B., Lange, K., and Weiner, D., *Am. J. Med. Sci.*, 1947, **213**, 61.

¹⁴ Rothlin, E., *Arch. Internat. de Pharmacodyn. et de Therap.*, 1923, **27**, 459.

¹⁵ Suzman, M. M., Freed, C. C., and Prag, J. J., *South African J. Med. Sci.*, 1938, **3**, 29.

¹⁶ Cobet, R., Ratschow, M., and Streckner, M. L., *Klin. Wchnsch.*, 1939, **18**, 278.

¹⁷ Ratschow, M., and Klosterman, H. C., *Z. f. Klinische Med.*, 1939, **135**, 198.

¹⁸ Loewe, L., and Rosenblatt, P., *Am. J. Med. Sci.*, 1944, **51**, 208.

¹⁹ Evans, J. A., and Boller, R. J., *J. A. M. A.*, 1946, **131**, 879.

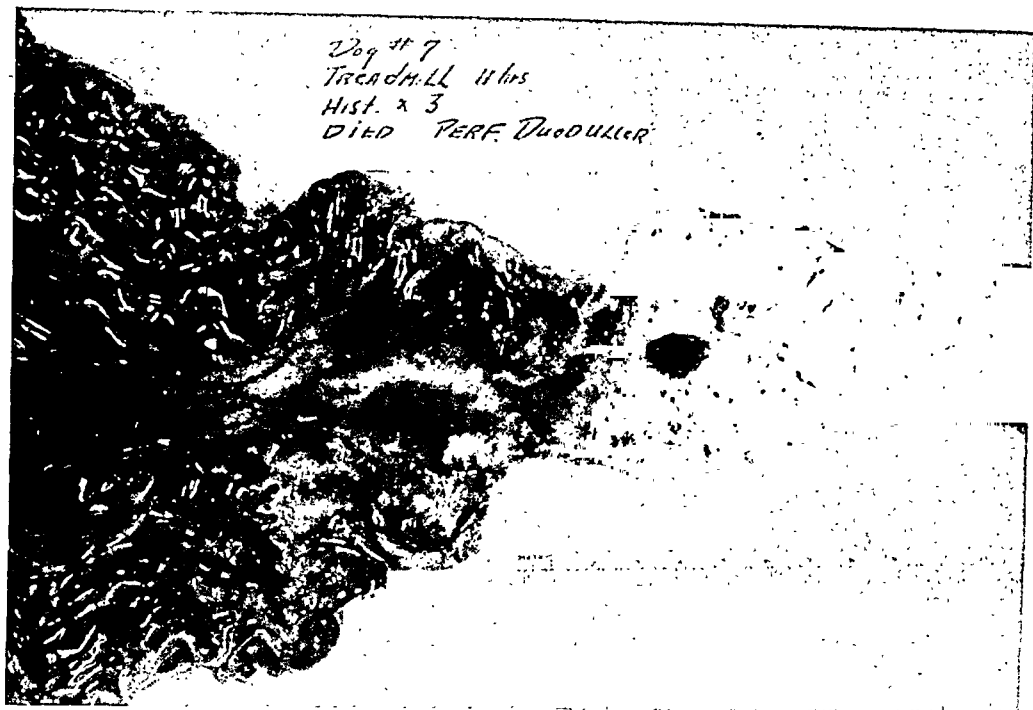


FIG. 1.

Stomach from dog No. 7, dying with a perforated duodenal ulcer after being run a total of eleven hours on the treadmill over a three-day period and at the same time receiving a daily injection of histamine-base.

erately severe muscular fatigue abets the histamine-provoked ulcer quite appreciably while at the same time depressing the output of hydrochloric acid. These seemingly paradoxical findings would suggest that the ulcer abetting effect of muscular fatigue is an effect on the mucosa itself (most probably on its blood supply) which renders it more vulnerable to the digestive action of the acid-pepsin mixture. There seems little reason to doubt that strenuous muscular exercise would cause a disturbance in the blood flow through the gastrointestinal tract by partitioning available blood volume in favor of the skeletal muscles. Evidence from this laboratory has shown that vasoconstriction³ produced by adrenalin-in-beeswax injection or by liberation of adrenalin due to exposure to cold⁴ may aid and abet the ulcer diathesis by creating such anoxic areas that fail to resist the digestive action of gastric juice. Muscular fatigue⁵ is likewise known to cause the liberation of adrenalin.

The greater weight loss in the experimental group (Table I) was due in part to the muscular exertion and in part due to the earlier development of an ulcer with consequent anorexia.

The hyperthermia associated with muscular fatigue may be a factor in the suppression of gastric secretion observed.

Conclusions. 1. Muscular fatigue in dogs abets the occurrence of the ulcer produced by chronic histamine stimulation.

2. Muscular fatigue in the dog depresses the output of hydrochloric acid in gastric juice from Heidenhain pouches both under basal conditions and after an artificial stimulation (aqueous histamine) due principally to a reduction in the volume of juice secreted.

³ Baronofsky, I., and Wangenstein, O. H., *Bull. Am. Coll. Surg.*, 1945, 30, 59.

⁴ Lillehei, C. W., unpublished data.

⁵ Gellhorn, E., *Autonomic Nervous Regulations*, Interscience Publishers, Inc., New York, N. Y., 1943, p. 135.

TABLE I.
Influence of Anticoagulants on Incidence of Ergotamine-Induced Gangrene in Rats.

Procedure	Total	Mortality		Survivors	Gangrene	
		No.	%		No.	%
Control	82	8	9.8	74	26	35.1
Heparin	28	5	17.9	23	9	39.1
Dicumarol	38	3	7.9	35	13	37.1
X ² *		1.19			0.012	
p†		>0.50			>0.99	
Significance		None			None	

* Corrected Chi Square.

† Probability.

TABLE II.
Usefulness of Fluorescein Test in Predicting the Development of Ergot Gangrene in Rats.

Results of test*	Ultimate incidence of gangrene					
	Group I (Tested 6 hr after ergotamine injection)			Group II (Tested 24 hr after ergotamine injection)		
	Total	Gangrene		Total	Gangrene	
		No.	%		No.	%
Fluorescence	11	4	34.9	18	5	27.8
No fluorescence	66	29	44.0	19	15	79.0
X ² †		0.019			7.79	
p‡		0.90			<0.01	
Significance		None			High	
Ass'n Coef.§		—			0.417	

* Presence or absence of fluorescence of tail 1 hr after injection of 1 cc 10% fluorescein.

† Corrected Chi Square.

‡ Probability.

$$\S = \sqrt{\frac{X^2}{n + X^2}}$$

2 rats, in which gangrene was developing in spite of therapy with dicumarol, revealed that the vessels were largely free of blood and there was no evidence of thrombosis. Similar studies on 2 control animals, developing gangrene, revealed accumulated masses of red cells containing small amounts of fibrin in the large arteries, but no actual thrombosis was observed. It is thus concluded that thrombosis is incidental, rather than contributory, to the vascular occlusion resulting in ergot gangrene and the severity of the vascular impairment is to be explained solely on the basis of the vasoconstriction produced by the ergot alkaloids.

In order to gain further information on the severity of the vascular impairment which can be produced by such vasoconstriction,

one cc of 10% sodium fluorescein was injected intraperitoneally into 12 normal and 23 ergotamine-treated rats. Fluorescence appeared in the ears of normal rats 2.7 ± 0.9 min. and in the tails 9.5 ± 1.91 min. after the injection of the dye. In the animals receiving ergotamine (12.5 or 25.0 mg/kg) the time required for the appearance of fluorescence in the ear was 9.32 ± 4.06 min. while in the tail 244 ± 182 min. was required. It is thus concluded that the circulation to the tail of rats receiving ergotamine is not only greatly, but preferentially impaired.

This preferential impairment of the circulation to the tail might be due either to the degree or the extent of the vasoconstriction occurring in this structure. In a series of ergotamine treated animals (37.5 mg/kg) no

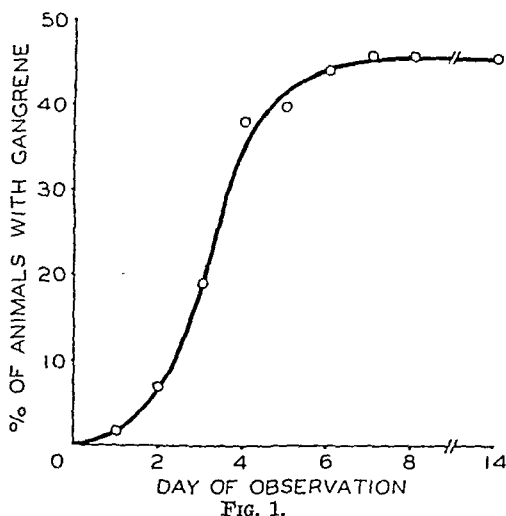


FIG. 1.

clotting time. This effect was maximum at 24 hours, and persisted for 36 hours. Clotting times were estimated by the capillary tube method on blood obtained from the tail. Following 3 daily doses of 30 mg of heparin in Pitkin's menstruum, the average clotting time exceeded 20 min. (normal = 1.04 ± 0.38 min.).

In view of the evidence that 50% of the control animals began to develop gangrene within 3 days of the injection of ergotamine, it was reasoned that therapy with anticoagulants need not be continued beyond this time. Thus, in the present series of animals, 5 daily doses of 30 mg/kg of heparin in Pitkin's menstruum were administered, beginning 2 days prior to the injection of ergotamine.

The incidences of actual necrosis in the series of 10, 8 and 5 heparin-treated rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 30.0, 50.0 and 40.0% respectively, and all animals which developed gangrene did so within 6 days of the injection of ergotamine.

Rats given 2.5 mg of dicumarol by mouth show changes in prothrombin times which are maximum at 24 hours and last 36 hours. These animals will tolerate 10 mg of dicumarol daily for 3 days.²⁰

²⁰ Overman, R. S., Stolman, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

Preliminary to the present study a series of rats were given 5 mg of dicumarol daily for 10 doses. Clotting times determined during this course of therapy were never above 8 min. However, bleeding from the tip of the tail after collection of blood for clotting determinations was greatly prolonged and 2 of 7 animals bled to death from this minor wound. Some animals developed spontaneous hemorrhages which stopped when dicumarol was discontinued and some showed severe anemia associated with tarry stools. The daily administration of 5 mg of dicumarol thus appeared to represent an effective and near maximum tolerated dose in the rat. In the present series 5 such daily doses were administered, beginning 2 days prior to the injection of ergotamine.

The incidences of actual necrosis in the series of 13, 16 and 6 dicumarol-treated rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 15.4, 31.3 and 100% respectively, and all animals which developed gangrene did so within 6 days of the injection of ergotamine.

Statistical analysis of the pooled data from the control, heparin and dicumarol experiments for the incidence of gangrene, as a function of the dose of ergotamine, revealed that no significant difference in the incidence of gangrene was attributable to the dose of ergotamine administered. Therefore, in evaluating the incidence of gangrene as a function of the treatment, the animals in the 3 dosage groups under any particular regime were pooled to give a figure representing the incidence for the group (Table I).

The incidences of gangrene in the control, heparin treated and dicumarol treated groups were 35.1, 39.1 and 37.1% respectively. Statistical analysis revealed no significant difference between these groups.

There would appear to be two possible explanations for the failure of heparin or dicumarol to reduce the incidence of ergotamine induced gangrene in the rat. They are (1) thrombosis does not contribute to the development of gangrene or (2) the anticoagulants employed were ineffective in preventing thrombosis.

Microscopic examination of the tails of

Effect of Intrasplenic Injections of Alloxan in the Rat.*

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Alloxan is usually effective in producing diabetes in experimental animals whether it is administered subcutaneously or intramuscularly,¹ intraperitoneally,² intravenously³ or enterally.⁴ This drug has a greater diabetogenic action when given to rats which have been fasted 2 or 3 days.⁵ Furthermore, the selective action of alloxan, in destroying certain cells of the pancreatic islets, takes place within a few minutes following its administration.^{6,7}

Since the action of alloxan is limited to such a short period of time following its administration, the present study has been undertaken in order to determine whether the liver might be responsible for the inactivation of this substance. Intrasplenic injections permit the alloxan to pass through the liver before it reaches the pancreas.

Material and Methods. One hundred and twelve adult male rats of the Long-Evans strain were used in this study. The animals were given intrasplenic injections of a 25% solution of alloxan and the dosages used were 50, 88, 125 or 150 mg/kg. The rate of injection, controlled by a micrometer syringe, varied in the different animals but the most satisfactory rate tried was 1/100 cc/min. Control rats received either intraperitoneal or sub-

cutaneous injections of alloxan in the same amounts and at the same injection rate as was injected intrasplenicly.

The rats were placed in metabolism cages for urine collections on the eighth and fifteenth days, respectively, following the injection of alloxan. Only rats with a 2-4+ urine sugar were considered to be diabetic. At the end of the 15-day period samples of tissue were taken for histological examination. Both the splenic and duodenal portions of the pancreas and pieces of liver were fixed in Bouin's solution.

Results. Considerable variation was seen in the response of the rats to the intrasplenic as well as the intraperitoneal injections of alloxan. Certain animals were not able to tolerate the toxic effects of the drug, regardless of the route of administration and differences in the injection rate and the amount of alloxan administered accounted for some variation. The data for the animals which were not fasted before they received the alloxan are shown in Table IA. The rate at which the injections were given is not indicated on the table. In most instances, however, the control rats received intraperitoneal injections of the same amount of alloxan, at the same rate, as did the animals which were given injections intrasplenicly. The smaller amounts of alloxan were usually more effective in producing diabetes when injected rapidly into the spleen than when given intraperitoneally. However, if 125 mg/kg were injected rapidly into the spleen, the animals usually died. Death in such cases was probably due, in many instances, to infarcts in the liver. The lobes of such livers showed a marked necrosis, although the crown region was generally normal.

Animals which received the drug intrasplenicly tolerated it much better when injections were made at a slower rate (1/100 cc/min.) but a given amount of alloxan was

* This study was aided by a grant from Hoffmann-LaRoche, Inc.

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⁵ Kass, E. H., and Waishren, B. A., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 303.

⁶ Hughes, H., Ware, L. L., and Young, F. G., *Lancet*, 1944, 1, 148.

⁷ Palay, S. L., and Lazarow, A., *Anat. Rec.*, 1946, 96, 55.

bleeding occurred when the tail was amputated within 1-2 cm of the base. Normal animals, so amputated, showed vigorous hemorrhage and even spurting of blood. The failure of the major arteries of the tail to bleed when transected implies practically complete closure of these vessels by ergotamine.

The time required for fluorescence to appear in the tail of rats receiving ergotamine is not to be construed as a measure of the duration of the action of ergotamine on this vascular bed. A highly diffusible substance, such as fluorescein, could slowly accumulate to a sufficient concentration to give fluorescence of the tissues, even in the presence of severe vasoconstriction. A different type of test was devised to give information as to the duration of the vascular impairment produced by ergotamine.

Seventy-seven rats were given fluorescein within 6 hours after having received ergotamine and another group of 37 rats were given the dye 24 hours after ergotamine. It was arbitrarily decided that all animals in which the time required for fluorescence to appear in the tail was longer than one hour should be classed as having a seriously impaired circulation. In the group tested within 6 hours after the administration of ergotamine 66 or 85.7% of the animals had seriously impaired circulation to the tail. In the group tested 24 hours after the administration of ergotamine 19 or 51.1% had seriously impaired circulation to the tail. Thus, in approximately half of the animals a single dose of 12.5-25.0 mg/kg of ergotamine can cause nearly complete vascular occlusion of the tail vessels, lasting for at least 24 hours.

It can be seen in Table II that a significant-

ly greater incidence of gangrene developed in those animals which, by the fluorescein test, still showed seriously impaired circulation to the tail 24 hours after the administration of ergotamine, than in those which did not. The coefficient of association between the performance on the fluorescein test carried out at 24 hours and the incidence of gangrene is of such magnitude (0.417) as to imply that the test is of considerable value in estimating the prognosis.

It may be assumed that an agent, to be effective against such gangrene, should be able to bring about fluorescence in a tail in which it did not appear as a consequence of the action of ergotamine. Prisol, papaverine, magnesium sulfate, aminophylline, nicotinic acid, alcohol, ether, demerol, histamine, methacholine and histidine-ascorbic acid were shown to be incapable of producing such an effect, and the tails of animals so treated remained nonfluorescent.

Summary. Twenty-four hours after the administration of a single dose of ergotamine tartrate, approximately 50% of rats showed, by means of the fluorescein test, a seriously impaired circulation to the tail, due to marked constriction of the major arteries of the tail. Forty to 50% of rats receiving a single dose of 12.5-37.5 mg/kg of ergotamine tartrate may be expected to develop gangrene of the tail and to begin to do so within one week of the injection of ergotamine. Anticoagulants, such as heparin and dicumarol, do not alter the incidence of ergotamine induced gangrene. It is concluded that the thrombosis, observed to be associated with this process by others, is incidental rather than contributory to the vascular occlusion.

Effect of Fagarine on Auricular Fibrillation.*

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In 1932 it was demonstrated that an alkaloid from an Argentinian plant, *Fagara coco*, exerts a depressant action on the heart.¹ Subsequent investigations showed that the purified alkaloid, fagarine, raised the fibrillation threshold during faradization of the ventricles² and that it may prevent the fibrillation which follows ligation of the coronary arteries.³

The action of fagarine on the established auricular fibrillation has not been tested because no method was available to elicit this disturbance for a period of sufficient duration without employing other measures which would influence the results. Post-faradization fibrillation is not stable and changes, as a rule, quickly into normal sinus rhythm. Auricular fibrillation persists for a longer time if faradization is combined with the administration of physostigmine, muscarine or choline derivatives. These substances, however, have side-effects on the cardiac muscle which make an evaluation of the experimental results difficult. If, however, 0.05 cc of a 0.05% solution prepared from aconitine crystals is injected subepicardially into the area of the sinus node in dogs it leads to auricular fibrillation or auricular flutter of long duration and permits the study of the effect of rapidly acting drugs. Such attacks of fibrillation occasionally last for more than an hour.⁴

Method. In 10 dogs the chest and the pericardium were opened in the usual way under

nembutal anesthesia and artificial respiration. Both vagi were severed in the neck in 7 experiments. The electrocardiogram was taken in Lead II. After the appearance of persistent auricular flutter or fibrillation 0.004 g/kg of alpha-fagarine chloride was injected intravenously.

Results. In all 10 experiments the flutter or fibrillation disappeared within one minute after the injection ended. Fig. 1a shows auricular fibrillation and its sudden change into sinus rhythm with a rate of 250 following an injection of 0.03 g of fagarine. In Fig. 1b auricular impure flutter changes into a sinus rhythm with a rate of 166. The sinus tachycardia following the disappearance of fibrillation is due at least in part, to the division of the vagi. In those experiments in which the vagi were intact the results were the same.

Table I gives the time intervals between the end of the intravenous injection of fagarine and the disappearance of the prevailing arrhythmia.

Following the injection of fagarine, faradic stimulation of the vagi, even with strong currents, had only a very slight effect on the cardiac rate and on a-v conduction. If larger doses of fagarine are used (0.01/kg) the faradic stimulation of the vagi is without effect.²

Inspection of the exposed heart showed no evidence of dilatation or any disturbance of contractility following the fagarine injection. This should be emphasized because definite dilatation and weakening of cardiac contractions does appear immediately if quinine or quinidine is injected in a dose sufficient to abolish fibrillation. A slight prolongation of the a-v conduction time, amounting to only 0.01-0.02 second appeared at the height of fagarine effect, and the RS-T segments were slightly depressed but no other changes were found in the electrocardiogram. This point

* Our thanks are due to the Laboratories Apotarg in Cordoba, Argentina, for the supply of the fagarine. This study was supported by a grant from Bernhard Altmann.

¹ Stueckert, G., and Sartori, A., *Rev. Univ. Nac. Cordoba, Argent.*, 1932, **10**, 12.

² Moisset de Espanes, E., and Moyano Navarro, B., *C. R. Soc. Biol.*, 1938, **127**, 510.

³ Moisset de Espanes, E., *C. R. Soc. Biol.*, 1938, **127**, 233.

⁴ Scherf, D., *Am. Heart J.*, in press.

TABLE I.
Effect of Alloxan Injections.

	Mg alloxan/kg body weight						
	A Non-fasted				B Fasted 2 days		
	50	88	125	150	88	125	150
A. Intrasplenic							
1. Diabetic	1	2	4			7	2
2. Non-diabetic	8	1	1	3	3	1	
3. Died	1	5	16	1			
B. Intraperitoneal							
1. Diabetic			7			6	2
2. Non-diabetic	4	4	6	2	3	4	
3. Died	2	3	3				
C. Subcutaneous							
1. Diabetic						8	
2. Non-diabetic						4	

less effective in producing diabetes.

A more uniform response was obtained and no deaths occurred when the rats were fasted 2 days before they were injected slowly with alloxan (Table IB). In the rats studied, 88 mg of alloxan per kg was not sufficient to elicit diabetes when given intrasplenically or intraperitoneally, while 150 mg resulted in a severe diabetes when injected by either method. When 125 mg/kg was given, diabetes was produced in 7 out of 8 rats when injected intrasplenically, in 8 out of 12 rats when injected subcutaneously, and in 6 out of 10 rats when given intraperitoneally. Within the limitation of the number of animals used in this study it was noted that diabetes in the latter group of animals was not so severe as in the other 2 groups.

The islets in both the splenic and duodenal portions of the pancreas were examined histologically in an attempt to determine whether one portion of this gland might be more susceptible to the alloxan. Islet damage, however,

was not localized in any particular region of the pancreas.

Comment. Since alloxan injected intrasplenically is just as diabetogenic as when given subcutaneously, indications are that this drug is not inactivated specifically in the liver. However, when administered intrasplenically, alloxan appears to be slightly more effective in producing diabetes than when given intraperitoneally. A possible explanation for this variation is that after the latter method of administration, the alloxan is absorbed over a large area of the peritoneal cavity and is thus diluted to a greater extent before reaching the pancreas.

Summary. The present observations indicate that alloxan is just as effective as a diabetogenic agent when given intrasplenically as when given subcutaneously. Similar amounts of alloxan given intraperitoneally result in many instances in a milder diabetes. It appears that alloxan is not inactivated specifically in the liver.

Enzyme Studies on the "Endocrine Kidney."

CHARLES D. KOCHAKIAN AND PAUL DONTIGNY.*

From the Department of Physiology and Vital Economics, University of Rochester, and the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal.

Selye¹ reported a technic for the transformation of the kidney into a purely endocrine organ in the rat. This technic consists in partially constricting the aorta between the origins of the 2 renal arteries by means of a ligature adjusted so that the blood pressure in the kidney below the constriction (in the rat the renal arteries leave the aorta at different levels) decreases to a point where no filtration occurs. To further insure against filtration taking place in that kidney the ureter is tied off and cut. The circulation of blood remains adequate, however, for the nutrition of the organ. Rats so treated have been shown to develop a very acute form of malignant hypertension.² The present experiment deals with the determination of the arginase and phosphatase content of these kidneys, which are non-functional as regards urine formation.

Procedure. Twelve female albino rats weighing between 150 to 160 g were used in our experiment. The operation as described by Selye and Stone² was performed under ether

anesthesia. Seven days later the animals were sacrificed and the kidneys removed, weighed and placed in separate vials containing 5 ml of distilled water; the right (normal) kidneys served as controls. Five samples were then sent fresh by air mail to Rochester where they were analyzed for the various enzyme activities by the previously described procedures.³⁻⁶

Results. There is a marked, indeed almost a complete, disappearance of both arginase and "alkaline" phosphatase from the "endocrine" kidney (Table I). The "alkaline" phosphatase was determined also with the addition of magnesium sulfate⁷ to the substrate mixture in order to rule out the possibility of lack of activator instead of a decrease in amount of this enzyme. The increase in activity was identical for the 2 sets of tissues, 47.7 and 46.7% for the "endocrine" and normal kidneys respectively. The change in "alkaline" phosphatase is in agreement with the observations by Wilmer⁸ on the kidneys of rabbits and rats after ligature of the ureters.

TABLE I.

Kidney	Wt, mg	Phosphatases					
		Arginase		"Alkaline"		"Acid"	
		Total U	U/g	Total U	U/g	Total U	U/g
Normal	811	64	99	109	141	15.0	18.2
Endocrine	357	6	17	5.5	16	6.0	16.6
% diff.	-56	-91	-83	-95	-89	-60	-8.8

U = Units.

* Fellow of the Canadian National Research Council.

The expenses of this investigation were defrayed through a grant from the Commonwealth Fund of New York (administered by Dr. H. Selye) and the Josiah Macy, Jr., Foundation (C. D. K.).

¹ Selye, Hans, *Nature*, July 27, 1946.

² Selye, H., and Stone, H., *J. Urol.*, 1946, 56, 399.

³ Kochakian, C. D., *J. Biol. Chem.*, 1944, 155, 579.

⁴ Kochakian, C. D., *J. Biol. Chem.*, 1945, 161, 115.

⁵ Kochakian, C. D., and Fox, R. P., *J. Biol. Chem.*, 1944, 153, 669.

⁶ Kochakian, C. D., *Am. J. Physiol.*, 1945, 145, 118.

⁷ Bodansky, O., *J. Biol. Chem.*, 1936, 115, 101.

⁸ Wilmer, H. A., *J. Exp. Med.*, 1943, 78, 223.

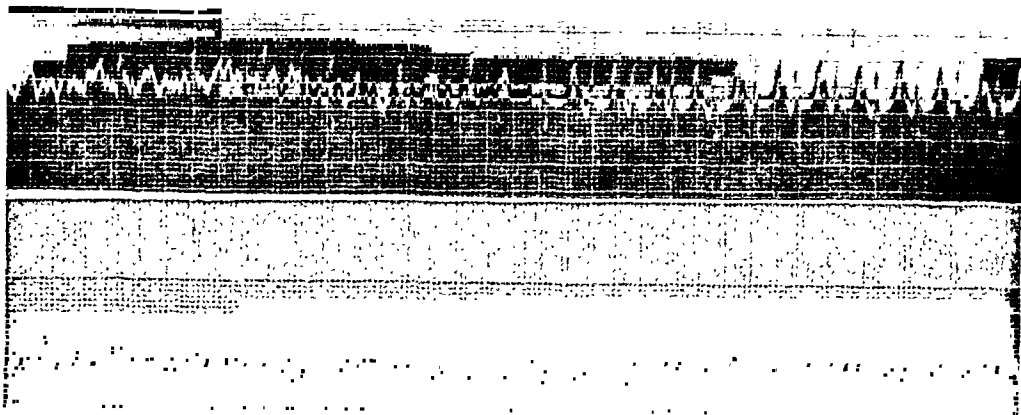


FIG. 1.

Fig. 1a (upper figure) shows the termination of auricular fibrillation and Fig. 1b (lower) the termination of auricular impure flutter by an intravenous injection of fagarine.

TABLE I.
Time of Disappearance of Arrhythmia.

Date	Prevailing arrhythmia	Time of disappearance of arrhythmia, seconds	Remarks
9/30	fibrillation	48	vagi intact
10/7	"	36	" severed
14	flutter	55	" intact
21	fibrillation	12	" severed
11/11	"	25	" "
25	"	18	" intact
1/7	"	25	" severed
2/26	"	22	" "
4/22	flutter	31	" "
5/13	fibrillation	42	" "

is also emphasized because the clinical administration of the drug occasionally leads to serious disturbances of stimulus formation in the ventricle.

The systolic blood pressure showed a temporary rise lasting only 2 to 5 minutes and amounting to 30-40 mm Hg. In all experiments the original values of blood pressure were registered within 5 minutes.

Following the injection of fagarine renewed

subepicardial injection of the aconitine solution had no effect on the existing sinus rhythm for about 45 minutes. After this time it was possible to elicit tachycardias and fibrillation for a second time with this method.

Summary. The intravenous injection of fagarine in dogs with an auricular flutter or fibrillation caused by aconitine abolishes these arrhythmias within one minute and leads to the reappearance of sinus rhythm.

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anesthesia. Seven days later the animals were sacrificed and the kidneys removed, weighed and placed in separate vials containing 5 ml of distilled water; the right (normal) kidneys served as controls. Five samples were then sent fresh by air mail to Rochester where they were analyzed for the various enzyme activities by the previously described procedures.³⁻⁶

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¹ Selye, Hans, *Nature*, July 27, 1946.

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⁷ Bodansky, O., *J. Biol. Chem.*, 1936, **115**, 161.

⁸ Wilmer, H. A., *J. Exp. Med.*, 1943, **78**, 225.

It is of special interest that the "acid" phosphatase, in contrast to the other enzymes, decreased in total amount but not in concentration (units per gram of tissue). It would seem, therefore, that this enzyme is necessary for the integrity of the cell and perhaps its endocrine function while the "alkaline" phosphatase and arginase are necessary only in

connection with urine formation.

Summary. In "endocrine kidneys" there is a marked indeed almost a complete disappearance of both arginase and "alkaline" phosphatase. On the other hand the "acid" phosphatase decreases in total amount but not in concentration.

16205

Activation of Hypertensin and Tyrosine by Subthreshold Amounts of Epinephrine.*

E. MYLON AND J. H. HELLER. (Introduced by M. C. Winternitz.)

From the Department of Pathology, Yale University School of Medicine.

Fractions of hog kidney containing renin do not produce constriction of the vessels of the rabbit's ear until subthreshold amounts of epinephrine are added to the perfusate.¹ Similar vaso-activation of liver fractions by epinephrine has been noted when they are combined with subthreshold amounts of epinephrine.² Fresh plasma is also capable of activating liver fractions.³ This last observation is of interest because of the report that vaso-inactive renin-containing kidney fractions become vasoconstrictive when plasma is added to the ear perfusate.⁴ This was ascribed to the formation of hypertensin rather than to the activation of renin by plasma. Since a similar constriction occurs following the addition of plasma to liver fractions under circumstances that would seem to be incompatible with the formation of hypertensin, a reinvestigation of the action of hypertensin on the vessels of the rabbit's ear was undertaken.

The experiments that follow show that hypertensin, a protein fraction, constricts the vessels of the rabbit's ear only when the perfusate is supplemented with traces of epinephrine or fresh plasma. This suggested the possibility that parts of the protein molecules, *c. g.*, peptides or even amino-acids, are responsible for the observed effect. Accordingly, the studies were extended to encompass a large majority of the known amino-acids. Special attention was directed to tyrosine, tyrosine-containing peptides, and a tyrosinamide in accord with Cruz-Coke's⁵ hypothesis that tyrosine molecules are necessary for the *in vivo* action of hypertensin.

Materials and Methods. Hypertensin was prepared from purified hog renin with the bovine globulin Fraction IV-1 (Armour) serving as substrate. Three hundred cc of a 1% solution of substrate were buffered at pH 7.5 and incubated for 10 minutes with 2-3 Swingle units of renin. N1 HCl was then added to bring the pH to 5.2 and the mixture immersed in boiling water for 10 minutes. The coagulated proteins were then removed by filtration and the almost water-clear filtrate lyophilized. The lyophilized material was taken up in 16 cc of distilled water and the small residuum of undissolved particles was

* Aided by a grant from the Commonwealth Fund.

¹ Mylon, E., Horton, F. H., and Levy, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 375.

² Mylon, E., Horton, F. H., and Levy, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 378.

³ Mylon, E., and Heller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 319.

⁴ Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, **71**, 29.

⁵ Cruz-Coke, E., *New York Academy of Sciences, Section of Biology, February 9 and 10, 1945; Science*, 1946, **104**, No. 2691.

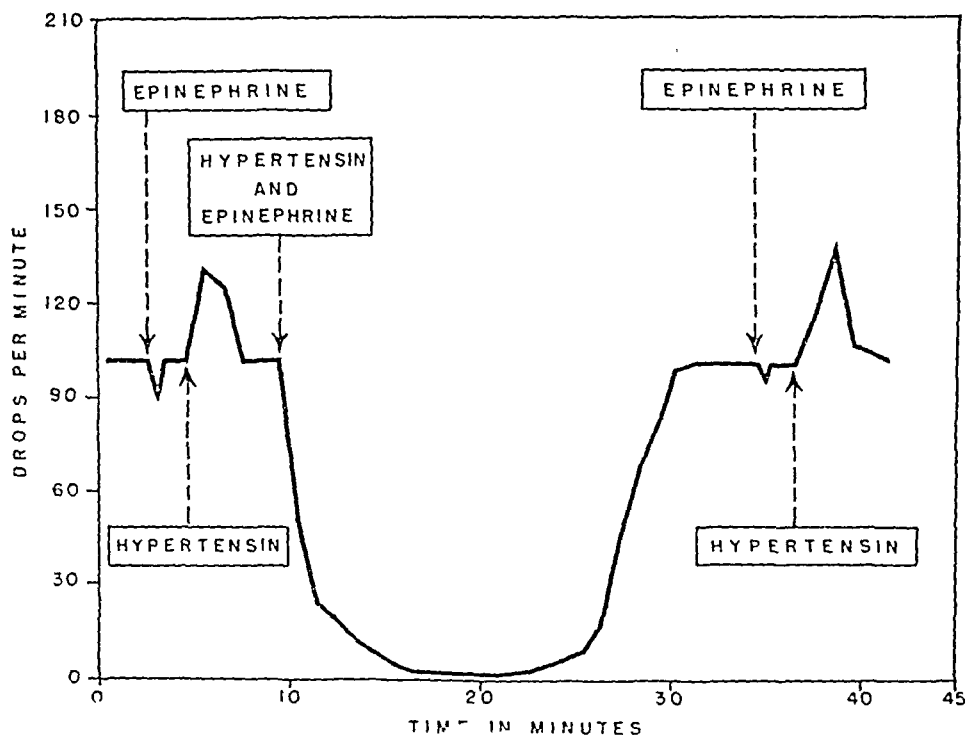


Fig. 1.

While the effect of 1 cc of epinephrine 1:5,000,000 is minimal and the effect of 1 unit of hypertensin is slightly dilating, combination of the two causes a strong and sustained constriction of the vessel of the rabbit's ear.

removed by centrifugation. Four cc of each lot was found to contain between 6 and 8 dog units of hypertensin as tested in the living animal. The remaining 12 cc of each lot were used in perfusion experiments, in amounts equivalent to from one-third to 2 dog units. The ear perfusion technic has been described.¹

Ears of young albino rabbits seemed to be more sensitive than those of older rabbits and of other types. A preparation was judged to be suitable when the initial drop rate per minute was between 100 and 160, and in addition when an injection of 1 cc of a 1:5,000,000 or 1 cc of a 1:10,000,000 solution of epinephrine caused minor and transient constrictions. Experiments were considered valid only when a terminal injection of epinephrine reproduced a reaction similar to the initial one. The order of the injections with the different materials was varied.

The plasma was prepared according to the

technic of Landis.⁶ The epinephrine used was Parke Davis 1:1000, and the dilutions were prepared as described previously.^{1,2}

The amount of an amino-acid, amide or dipeptide used for a single injection was between 1/100 and 1/200 of a millimole; for *l*-tyrosine it was always 1/200 of a millimole. The pH of the Ringer-Locke solution (7.3) was not changed by the addition of the small amounts of the test substances.

The following amino-acids were used: *dl*-phenylalanine, *dl*-alanine, *dl*-serine, *l*-valine, glycine, *l*-cystine, *l*-tryptophan, *dl*-glutamic acid, *l*-aspartic acid, *dl*-lysine, *l*-leucine, *dl*-isoleucine, *dl*-norleucine, *dl*-threonine, *l*-proline, *l*-tyrosine, *l*-hydroxyproline, *l*-histidine, *dl*-arginine and *dl*-methionine.

The tyrosine-containing dipeptides glycyl-*l*-tyrosine and *l*-tyrosyl-glycine, and also the

⁶ Landis, E. M., Wood, J. E., Jr., and Guerrant, J. L., *Am. J. Physiol.*, 1943, 139, 26.

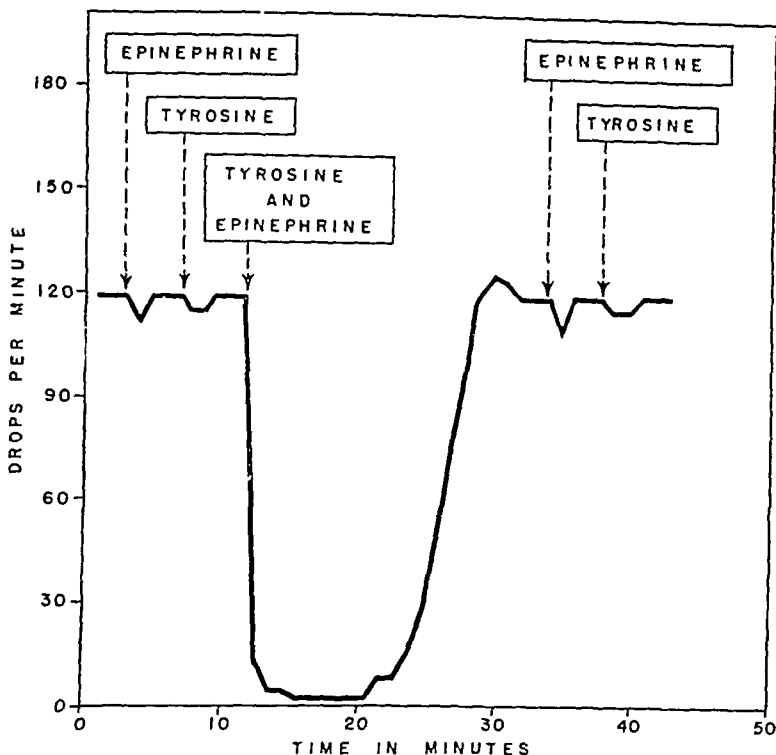


FIG. 2.

Insignificant effect of epinephrine and *l*-tyrosine alone in contrast to the strong vasoconstriction elicited by a combination of both.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.

l-Tyrosine: 0.005 millimole.

tyrosine-amide acetate were tested.[†]

Iodine was introduced into the tyrosine molecule by slowly adding *n*/10 aqueous iodine solution to *n*/1000 solution of *l*-tyrosine until a faint yellow color remained. After keeping the mixture in the dark for several hours, the excess iodine was removed by heating.

Results and Brief Discussion. *Hypertensin alone:* Neither the initial injection of hypertensin nor its subsequent introductions into the perfusion circuit produced vasoconstriction as determined in numerous experiments with 15 different lots. A slight vasodilation was the usual sequel of the addition of hypertensin to the perfusion fluid.

Hypertensin plus epinephrine: On the

other hand, when hypertensin was combined with subthreshold amounts of epinephrine, powerful and sustained vasoconstriction followed. A typical result can be seen in Fig. 1. A total of 28 perfusion experiments were carried out to test the effect of the combination of hypertensin with subthreshold amounts of epinephrine. The vasoconstriction that followed was so intense in 14 instances that the vessels of the ear reduced the drop rate 90 to 99% for 4 to 18 minutes and 100% for 20 minutes. In 10 of the other 14 tests the drop rate was reduced 60 to 90% for from 3 to 8 minutes and in the remaining 4 experiments a more moderate reduction of 50% was observed, lasting from 1 to 8 minutes.

Hypertensin plus plasma: When 1 cc of fresh rabbit plasma was used in place of epinephrine in combination with hypertensin,

[†] We are indebted to Dr. J. S. Fruton for the generous supply of glycyl-*l*-tyrosine (*l*-tyrosyl-glycine), and tyrosine-amide-acetate.

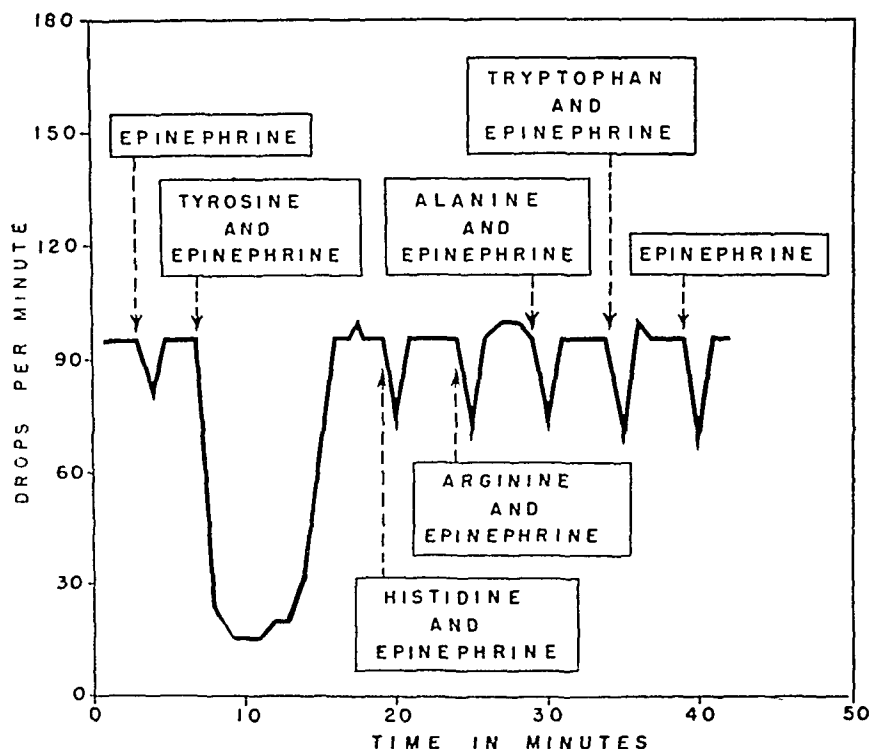


FIG. 3.

Strong vasoconstriction is solely elicited by *l*-tyrosine plus epinephrine and not by a combination of other amino acids and epinephrine.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.

l-Tyrosine: 0.005 millimole.

The other amino acids: 0.01 millimole.

a strong vasoconstriction was elicited. This effect was only obtainable with fresh plasma and incubating the plasma for 8 hours abolished its ability to activate hypertensin. These results are analogous to those previously reported, in which liver fractions were combined with fresh and incubated plasma.³

On the basis of the identical results obtained with plasma plus renin⁴ and plasma plus hypertensin, it could not be decided whether the observed constriction followed a direct action of plasma on renin or an action of plasma on hypertensin. The specificity of the renin-hypertensinogen reaction on the other hand suggested additional experiments in which it was found that renin itself is being activated. Vasoinactive pig renin, unable to catalyze hypertensin formation with human hypertensinogen, in combination with vasoinactive human plasma produced a significant

constriction in the vessels of the rabbit's ear.

Amino-acids: A total of 95 experiments involving one or another of 20 different amino-acids failed to demonstrate any effect when these were added individually to the rabbit's ear perfusate. More important is the fact that with one exception, tyrosine, there was only an inconstant, slight and transient response, for any one compound when traces of epinephrine were added to the perfusate. Addition of epinephrine to the tyrosine-containing perfusate was followed by a sharp and sustained decrease in the drop rate, 45% to 90% for from 3 to 9 minutes (Fig. 2 and 3).

These findings led to the exploration of other tyrosine-containing compounds including glycyl-*l*-tyrosine, *l*-tyrosyl-glycine, and tyrosine-amide-acetate. Needless to say they had no influence when added to the Ringer-Locke perfusate. This status persisted when

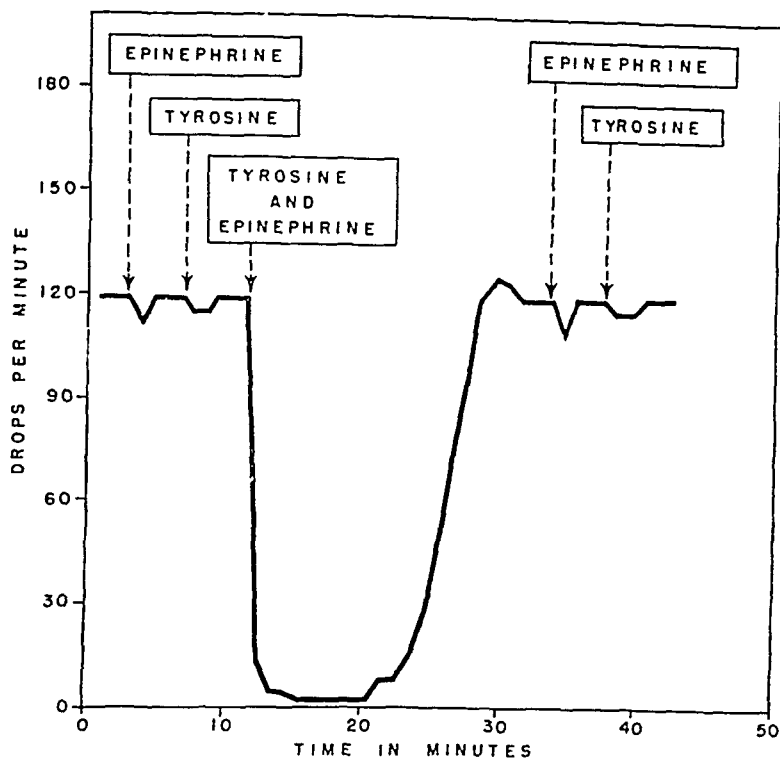


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[†] We are indebted to Dr. J. S. Fruton for the generous supply of glycyl-*L*-tyrosine (*L*-tyrosylglycine), and tyrosine-amide-acetate.

Hypertensin plus plasma: When 1 cc of fresh rabbit plasma was used in place of epinephrine in combination with hypertensin,

tyrosine molecule abolished the constrictor action entirely.

The conclusion drawn from these findings is that constriction of the blood vessels of the

rabbit's ear is dependent to a large degree upon traces of epinephrine and tyrosine-containing compounds of special configuration.

16206

Prevention of Experimental Dietary Hepatic Cirrhosis by Goitrogenic Substances.

PAUL GYÖRGY, CATHARINE S. ROSE, AND HARRY GOLDBLATT.

From the Department of Pediatrics and the Department of Medicine, School of Medicine, University of Pennsylvania, Philadelphia, and the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

In previous observations¹ thiouracil, and to some extent thiourea, exerted a beneficial effect in the prevention of dietary cirrhosis of the liver in rats. In animals receiving thiourea the food intake was generally very low and their weights showed a rapidly progressive decline. In contrast, the general well being of rats fed the same basal ration mixed with thiouracil (0.1% in the diet) remained undisturbed. The weight losses sustained were smaller than in the control animals without any appreciable difference in the average food intake in the two groups.

The beneficial effect of thiourea and thiouracil in the prevention of experimental dietary cirrhosis has been related to their effect on thyroid function, specifically to the decreased metabolic rate including the metabolism of protein. Decreased turnover of proteins pertains also to that of their constituents, including methionine, which is one of the key substances in the pathogenesis of dietary hepatic injury.²

It is difficult to furnish direct proof for the correctness of this assumption. The demonstration of strict parallelism between goitrogenic activity and preventive effect on hepatic cirrhosis may serve as indirect circumstantial evidence. The superior effect of thiouracil over thiourea is in good accord with these

considerations. In this connection it seemed advisable to include propyl-thiouracil and aminothiazole in these investigations. Propyl-thiouracil is at present probably the most effective goitrogenic agent available. Aminothiazole, when first used, was reported to be very effective in the treatment of hyperthyroid conditions.³ However, pharmacological⁴ and more recent clinical studies⁵ are at variance with this conclusion and support the view that aminothiazole is less effective and more toxic than propyl-thiouracil or even thiouracil.

Experimental. During the course of our studies extending over several years it became evident that even without any change in the basic composition of the experimental cirrhosis-producing diet the results, such as incidence and severity of cirrhosis, food intake, weight changes, and survival time, may vary at various times. These variations—admittedly within narrow limits—are probably due to changes in the composition of the fat used (perhaps in addition to obscure climatic influ-

³ Perrault, M., and Boyet, D., *Annales d'Endocrinologie*, 1945, **5**, 86; *Lancet*, 1946, **1**, 731; Boyet, D., Bablet, J., and Fournel, J., *Ann. de l'Inst. Pasteur*, 1946, **72**, 165.

⁴ Astwood, E. B., Bissell, A., and Hughes, A. M., *Endocrinology*, 1945, **36**, 456.

⁵ Morgans, M. E., *Lancet*, 1947, **1**, 519; Williams, R. H., *Arch. Int. Med.*, 1947, **80**, 11; McConnell, J. S., Frost, J. W., Wilber, R. W., and Rose, E., *Ann. J. Med. Sc.*, in press.

¹ György, P., and Goldblatt, H., *Science*, 1945, **102**, 451.

² See literature. György, P., *Am. J. Clin. Path.*, 1944, **14**, 67.

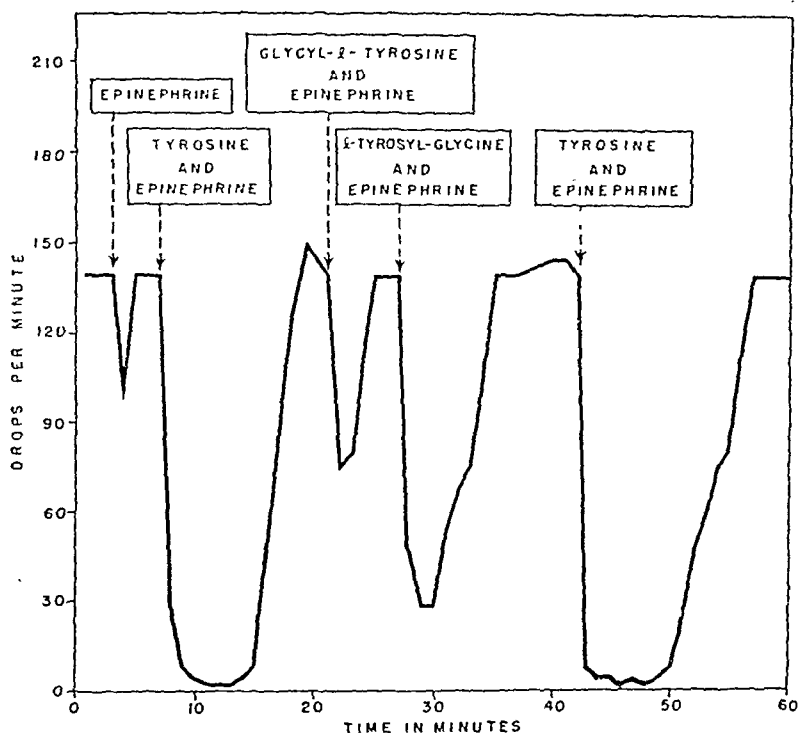


FIG. 4.

The constrictor effect of *l*-tyrosine plus epinephrine is much greater than that of *l*-tyrosyl-glycine plus epinephrine. The effect of glycyl-*l*-tyrosine and epinephrine is insignificant.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.

l-Tyrosine: 0.005 millimole.

The dipeptides: 0.01 millimole.

glycyl-*l*-tyrosine was supplemented with traces of epinephrine. On the other hand, *l*-tyrosyl-glycine and tyrosine-amide-acetate combined with minimal amounts of epinephrine had a moderate constrictive effect on the vessels of the rabbit's ear (Fig. 4).

The vasoconstrictive effect of tyrosine, it would seem, is abolished when the amino group is linked to a carboxyl of a second amino acid. When the amino group remains free and the carboxyl group is bound by another amino acid or transformed into an amide, the vasoconstrictive effect persists to about 50%.

Another finding still difficult to correlate with the coverage of the amino group of tyrosine-like compounds is the effect of iodination. This also abolished the vasoconstrictive effect. These results are of interest in relation to Cruz-Coke's suggestions⁷ that the pressor action of hypertensin *in vivo* might

be related to its tyrosine content.

Summary. Hypertensin has no effect on the vessels of the perfused rabbit's ear. It becomes strongly vasoconstrictive, however, when supplemented with traces of epinephrine and also when fresh plasma is added to the perfusate. None of 20 different amino acids tested in perfusion experiments on the rabbit's ear had any constrictor effect. When traces of epinephrine were included in the perfusion fluid, tyrosine was the only one of the 20 that exhibited a strong and sustained vasoconstriction. Glycyl-*l*-tyrosine was inactive with and without epinephrine. *l*-tyrosyl-glycine and tyrosine-amide-acetate, both inactive when perfused alone, constricted the vessels if combined with minute amounts of epinephrine. The activity of these two compounds, however, was definitely less than that of tyrosine. Introduction of iodine into the

absence of serous (partly chylous) effusions in the peritoneal, pleural, and pericardial cavities, in far better survival rate and in more satisfactory weight curves. It was especially impressive to the observer that these better weight curves were obtained with a food consumption only 52% of that of the control animals fed the same diet without propyl-thiouracil. The animals receiving propyl-thiouracil exhibited a perfect fur and were altogether in an exceptionally good general condition.

As would be expected, in the absence of cod liver oil⁶ and in the presence of hydrogenated fat (Crisco),⁷ ceroid was found in the livers even of the control animals in only limited quantity or not at all. Even these irregularly occurring traces of ceroid were missing in the animals fed the diet containing additional propyl-thiouracil. In contrast, the fat content of the liver judged on the basis of histologic examination and not by the more reliable chemical analytical data appeared to stay elevated in both the control animals and in the experimental animals receiving propyl-thiouracil.

The incidence of acute or subacute necrotizing nephrosis² happened to have been low in the present group of control animals, only 2 rats out of 12 showing these changes. In the light of this low incidence in the control group, the fact that no renal changes were observed in the group of rats receiving propyl-thiouracil is of interest but of no statistical significance. Atrophy of the testes with suppressed spermatogenesis was seen in 7 out of 10 rats in the control group (A) and in 8 out of 10 in the corresponding group (B) fed propyl-thiouracil. Thus, the injurious effect of the diet on the gonads remained uncorrected by propyl-thiouracil.

In contrast to the remarkable preventive effect of propyl-thiouracil as documented by the data of Exp. I (Table I), aminothiazole in the dose given (0.05%) seemed to influence only very slightly, if at all, the production of

dietary hepatic injury in rats (Exp. II, Table I). The slight reduction in the incidence of cirrhosis in the group of rats fed aminothiazole might indicate some beneficial effect but it is of no statistical significance. The only significant change brought about by aminothiazole was with regard to the occurrence of effusions in the various serous cavities: instead of 10 animals out of 15 in the control group (A), only 2 out of 15 in the experimental group (B) exhibited such pathologic manifestations.

Again, in contradistinction to the effect of propyl-thiouracil, aminothiazole exerted no significant influence on ceroid production or on food intake, survival rate, and weight changes of the experimental animals. The liver fat estimated histologically appeared to be high in both the experimental (B) and control animals (A). There was no difference in the incidence of renal or testicular changes between the groups not receiving and those receiving aminothiazole.

Discussion. The present and previous¹ observations are in good agreement regarding the beneficial effect of goitrogenic substances on the prevention of dietary cirrhosis of the liver in rats. The parallelism between goitrogenic and "anti-cirrhotic" potency is satisfactory. Propyl-thiouracil which is considered the most powerful goitrogenic substance proved also to be superior to the other goitrogenic substances tested in its effect on the prevention of dietary cirrhosis of the liver. As regards their relative effectiveness against cirrhosis these substances are in the following order: Propyl-thiouracil > Thiouracil > Thiourea > Aminothiazole. Of course this order applies only to the quantitative conditions chosen for the present and previous experiments. Nevertheless it is of interest to note that on the basis of food intake and molecular weight propyl-thiouracil appears to be at least 5 times as potent in its effect on the prevention of hepatic cirrhosis as thiouracil. The daily dose of propyl-thiouracil amounted on the average to 1.95 mg, which in itself is good support for the assumption that propyl-thiouracil and other goitrogenic substances act with greatest probability through the intermediary of the thyroid gland. So-called lipo-

⁶ Endicott, K. M., *Arch. Pathol.*, 1944, **37**, 49; Endicott, K. M., Daft, F. S., and Sebrell, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 330; Wachstein, M., *ibidem*, 1945, **59**, 73.

⁷ György, P., unpublished observations.

ences), all other experimental conditions being fairly strictly controlled. These observations necessitate the liberal use of controls in experiments in which the effect of a particular substance on the production of dietary hepatic injury is being investigated. In the present report the effect of 6-propyl-thiouracil and of 2-aminothiazole has been studied in two consecutive experiments. In each experiment one group of control animals was placed on the basal experimental ration without goitrogenic substance, to be compared with a second test group of rats receiving the same basal diet supplemented with propyl-thiouracil or aminothiazole. Young adult male rats with an initial weight of 150-225 g were used throughout.

The basal diet consisted of casein (G.B.I.) 8, Crisco 40, sucrose 48, salt mixture 4, with the admixture of 0.05% propyl-thiouracil or 0.05% aminothiazole in the corresponding group of each experiment. Each animal received daily 20 μ g thiamine chloride, 25 μ g riboflavin, 20 μ g pyridoxine, and 100 μ g calcium pantothenate, all dissolved in 1 cc of water. Three drops of percomorph oil furnishing 3750 units of vitamin A and 540 units of vitamin D, and 3 mg of mixed tocopherols (Distillation Products) were given weekly. In addition all animals received 50 mg of cystine daily with the purpose of accelerating and aggravating the cirrhotic changes in the liver. As customary, the experimental period was 150 days and those rats that survived were then killed. The final diagnosis of hepatic injury was based, without exception, on microscopic examination. Minor but definite cirrhotic changes were marked with +, whereas more severe cirrhosis was defined as ++ to +++++, with proper consideration for its varying degree in different lobes.

Results. From the tabulation of all the pertinent data collected (Table I, Exp. I) it becomes evident that propyl-thiouracil when added to the basic cirrhosis-producing synthetic diet exerted a marked preventive effect, manifesting itself not only in much lower, almost negligible incidence (8 out of 10 experimental animals were free from cirrhosis) and in milder degree of cirrhosis, but also in

TABLE I.
Effect of Propyl-thiouracil and Aminothiazole on Body Weight, Food Intake, and Hepatic Injury in Rats Fed a Cirrhosis-Producing Diet.

Exp.	Supplement	No. of animals	Wt loss		No wt loss	Avg wt loss, %	Dead before 150 days	Killed at 150 days	Fluid in serous cavities	Food intake, g	Cirrhosis			Ceroid	
			>50 g	<50 g							0	+	++ to +++++	0	Trace to +
I.	A. None	12	8	3	1	-29.6 \pm 21.7	6	6	5	7.5 \pm 0.95	0	1	11	6	6
	B. Propyl-thiouracil	10	0	9	1	-11.0 \pm 5.1	0	10	0	3.9 \pm 0.23	8	1	1	10	0
II.	A. None	15	3	3	9	-8.4 \pm 26.0	4	11	10	5.8 \pm 0.93	1	1	13	10	5
	B. Aminothiazole	15	2	8	5	-7.1 \pm 20.5	2	13	2	6.0 \pm 0.62	4	2	9	5	10

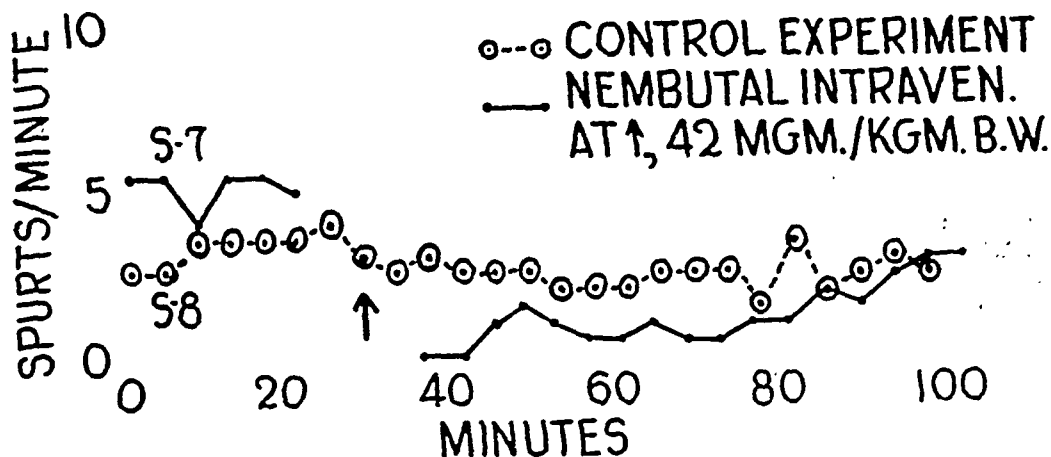


FIG. 1.
Ureteral Peristalsis in Unoperated Dog.

animals. Trattner's method resulted in a more physiologic approach to the study of ureteral peristalsis in that anesthesia and operative manipulation were eliminated. The present work likewise eliminated all operative trauma, and also anesthesia except in those experiments in which the effects of the anesthetic *per se* were being investigated. It goes one step further to realize the ideally physiologic experiment in that no catheter is introduced into the ureter. Although the presence of a catheter in the lower end of the ureter may have very little, if any, effect on the activity of the upper part of the structure, the absence of the catheter is a closer approach to the normal state.

Materials and Procedure. Well-trained female dogs, weighing approximately 11 kilograms, were loosely restrained lying supine on a dog-board. The animals had been without food for 24 hours but had free access to water. The urinary bladder was catheterized with a hard-rubber catheter and was washed free of urine with distilled water. This preliminary catheterization dilated the urethra so as to facilitate the passage of the cystoscope (No. 16 Fr. children's cystoscope). After several washings of the bladder the catheter was removed and the cystoscope introduced into the bladder without difficulty. No local anesthetic was used. One cubic centimeter of a sterile 4% aqueous solution of indigo carmine was injected intramuscularly. Three to fifteen minutes later the ejection of the dye from

the ureteral orifice was observed. Although only one ureteral orifice could be watched because of the limited visual field of the cystoscope, in a few experiments the 2 ureteral orifices were so located that the spurting of dye from both could be observed simultaneously. (The curves and the table give data for one ureter in each experiment, however.) The bladder was always distended with about 50 cc of water to facilitate the observations of the spurts of dye.

After the dye entering the bladder became distinctly visible, the number of spurts of dye was counted, usually for a 2-minute period, followed by a 2-minute rest period for the observer. This alternation of counting and rest periods was continued throughout the experiment. If the dog was restless the counts were made for one minute, followed by a 2-minute rest period. The dye was frequently washed from the bladder with distilled water through the cystoscope, the water entering from a reservoir suspended above the animal. Only the observers who began the experiment and who were known by the animals were allowed in the room during the experimental period on the unanesthetized animals so as to minimize emotional disturbance of the animals.

The cystoscopic observation of dye entering the urinary bladder of unanesthetized dogs was reported by Milliken and Karr² in their studies

² Milliken, L. F., and Karr, W. G., *J. Urol.*, 1925, 13, 1.

tropic substances, such as methionine or choline, used for the prevention of hepatic cirrhosis, require relatively higher doses to be effective.

Aminothiazole was found to be without any significant benefit in doses which on the basis of food intake and molecular weight were about 3.5 times higher than those used for propyl-thiouracil.

Successful prophylaxis of hepatic cirrhosis with goitrogenic substances such as thiouracil in the previous¹ and with propyl-thiouracil in the present studies does not necessarily lead to the conclusion that the same approach may be successful in the therapy of this condition. As a matter of fact, in unpublished experiments thiouracil appeared to exert no effect or even a slightly deleterious one on a *pre-existing* cirrhosis in rats.

As an unexpected indirect result of our studies the excellent general nutritional state of the experimental animals receiving propyl-thiouracil deserves special attention. This observation supports the view expressed in another connection by several authors⁸ that goitrogenic substances could play an important role in the feeding and fattening of farm animals, and especially in the feeding efficiency, an all-important economic factor. Older experiments were carried out mainly with thiouracil. Our own studies seem to give propyl-thiouracil a definite edge over thiouracil. The practical problem as to whether goitrogenic substances are stored and, if so, how long in animals receiving them with their feed, and whether the ingestion of such

animal products may exert a toxic (goitrogenic) effect on the consumer is beyond the scope of this presentation.

Summary. Admixture of propyl-thiouracil (.05%) with a cirrhosis-producing synthetic diet will prevent effectively the production of dietary cirrhosis in rats. This result is achieved with simultaneously reduced food intake and is manifested not only in the absence of cirrhotic changes in the liver but also in the improved general condition of the experimental animals and increased feeding efficiency.

Aminothiazole in the dose used (3½ times the equivalent of propyl-thiouracil) was without any significant effect on the production of dietary cirrhosis, on food intake, survival rate and weight changes.

For the goitrogenic substances tested efficiency in prevention of cirrhosis seems to parallel goitrogenic potency.

⁸ Kempster, H. L., and Turner, C. W., *Poultry Sc.*, 1945, **24**, 94; Andrews, F. N., and Schnetzler, E. E., *ibid.*, 1946, **25**, 124; Glazener, E. W., and Jull, M. A., *ibid.*, 1946, **25**, 236; Mixner, J. P., Tower, B. A., and Upp, C. W., *ibid.*, 1946, **25**, 536; Reineke, E. P., Davidson, J. A., Wolterink, L. F., and Barrett, F. N., *ibid.*, 1946, **26**, 410; Andrews, F. N., Beeson, W. M., Herriek, E. R., and Harper, C., *J. Animal Sc.*, 1947, **6**, 3; Beeson, W. M., Andrews, F. N., and Brown, P. T., *ibid.*, 1947, **6**, 16; Muhrer, M. E., and Hogan, A. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 211; Van der Noot, G. W., Reece, H. P., and Skelley, W. C., *J. Animal Sc.*, 1947, **6**, 12; McMillen, W. N., Reineke, E. P., Bratzler, L. J., and Francis, M. J., *ibid.*, 1947, **6**, 305.

16207 P

Observations on Ureteral Peristalsis in Unoperated Dogs.

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Up to the time Trattner^{1,2} developed the hydrophorograph for the graphic recording

of peristaltic activity in the human ureter, investigations on ureteral peristalsis were carried out on excised ureters, or on exposed ureters *in situ* in anesthetized and operated

¹ Trattner, H. R., *J. Urol.*, 1924, **11**, 477.

² Trattner, H. R., *J. Urol.*, 1932, **28**, 1.

16208 P

Inhibition of Typhus and Spotted Fever by Intradermal Inoculation of Antiorgan or Certain Normal Sera.*

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The present report includes one phase of the study on the mechanism of action of antiorgan sera (ACS=REIS) on infectious processes,¹ namely: on the course of typhus and spotted fever[†] in guinea pigs after intradermal inoculation into an area previously infiltrated with the antiserum. This involves primarily the role of the skin as a natural portal of entry and as dermal barrier for these infections.

Methods. A square area (1 cm along each side) on the shaved abdominal skin of the guinea pig is delineated and injected intradermally at 4 corners toward the center with the serum in 1:5 or 1:10 dilutions (0.1 ml at each corner). The center of this area is injected 2 hours later with 0.1 ml of a 10% brain suspension of a typhus or spotted fever guinea pig (Anigstein *et al.*²) The antiorgan serum was prepared in rabbits with guinea pig spleen and bone marrow as antigen according to the original technic of Marchuk,³ and its modifications (Anigstein *et al.*⁴). Each series of test guinea pigs was accompanied by controls injected either with infective brain tissue alone, or in addition, with normal

rabbit serum substituted for the immune serum. The potency of the antisera was based on complement fixation titers with homologous antigen (spleen and bone marrow), and by action on the outgrowth of tissue explants (Pomerat⁵). In one series, the globulin fraction of the antiginea pig serum in dilution 1:10 was used. Absorption tests with boiled sheep cells of both antiorgan and "normal" rabbit sera showing antisheep hemolysins were carried out to study the possible role of the Forssman antibodies. Groups of 6 to 12 afebrile guinea pigs of both sexes (400-500 g body weight) were used in each series of experiments involving various combinations of the test sera. Body temperatures of test guinea pigs were recorded daily over a period of 18 days.

Results. The first series of 6 guinea pigs were treated with antiginea pig rabbit serum (dilution 1:10) and 2 hours later injected at the site of serum inoculation with the brain suspension of a typhus infected guinea pig. Of these, 4 animals remained afebrile; in one the course of the disease was markedly attenuated, and one showed typical typhus. All controls reacted with typical fever after 6 days incubation. Of 6 test animals in another series, 5 remained afebrile, while only one responded with fever of 2 days duration. Significant results were obtained with antiorgan serum No. 91 (complement fixation titer 1:150; hemolysin titer 1:1000) used in a dilution 1:5. Of 5 guinea pigs, 4 remained afebrile, one developing fever after 11 days. The controls treated with serum (diluted 1:5) from the same rabbit (No. 91) bled before immunization ("normal" serum), reacted with typical typhus.

Occasionally, sera from untreated ("normal") rabbits attenuate the course of the

* Study aided by grants from the John and Mary R. Markle Foundation and from the Lilly Research Laboratories.

† The strain of louse-borne typhus was secured through the courtesy of Dr. John P. Fox, Laboratories of the International Health Division, Rockefeller Foundation, New York. The Rocky Mountain spotted fever strain was kindly supplied by Dr. R. R. Parker, Director, Rocky Mountain Laboratory, U. S. Public Health Service.

¹ Anigstein, L., and Pomerat, C. M., *Tex. Rep. Biol. Med.*, 1945, **3**, 545.

² Anigstein, L., *et al.*, *J. Immunol.*, 1944, **48**, 69.

³ Marchuk, P. D., *Am. Rev. Soc. Med.*, 1943, **1**, 113.

⁴ Anigstein, L., *et al.*, *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 279.

⁵ Pomerat, C. M., unpublished data.

URETERAL PERISTALSIS IN UNOPERATED DOGS

TABLE I.
Effects of Anesthesia on Ureteral Peristalsis.

Experiment No.	Ureteral peristaltic activity—per minute				
	Pre-experimental		Experimental (Intravenous nembutal)		
	Period 1	Period 2	Period 3	Period 4	Period 5
S-7 (42 mg/kg)		5.6 (4.5-6.0)	1.2 (0.5-2.0)	1.1 (1.0-1.5)	2.1 (1.5-3.0)
S-5 (30 ")	4.2 (3.5-6.5)	4.3 (3.5-5.5)	4.7 (3.5-8.0)	4.1 (3.5-4.5)	4.0 (3.5-4.5)
S-4 (29 ")	4.8 (3.0-6.5)	5.0 (3.0-6.0)	2.2 (2.0-2.5)	2.7 (2.5-3.0)	2.9 (2.0-4.0)
S-5 (27 ")		3.8 (3.0-4.5)	2.0 (1.5-2.5)	3.5 (1.5-4.5)	
Controls					
S-8	3.6 (3.0-4.0)	3.7 (3.0-4.5)	2.8 (2.5-3.0)	2.7 (2.0-3.0)	3.2 (2.5-4.0)
S-6	5.7 (4.5-7.0)	4.3 (3.5-5.5)			
S-2	2.7 (2.0-3.0)	3.1 (2.5-4.0)			
S-1	3.1 (2.0-4.0)	2.5 (1.5-3.5)	2.6 (0.5-4.5)		
B-6 (42 mg/kg)		4.6 (4.0-5.0)	5.8 (5.0-8.5)	4.2 (3.5-5.0)	3.9 (2.5-4.5)
B-3 (28 ")		5.7 (5.0-7.0)	4.2 (3.5-5.0)		
B-5 (25 ")	4.7 (4.0-5.5)	7.2 (6.0-9.5)	4.9 (3.5-6.0)	3.9 (3.5-5.0)	
B-4 (25 ")		4.7 (4.5-5.0)	5.3 (3.0-10.5)	4.6 (4.0-6.0)	4.1 (3.0-5.0)
B-2 (25 ")		4.9 (4.5-5.5)	3.5 (2.5-5.0)		
Controls					
B-7	7.4 (5.0-9.0)	8.9 (6.5-10.0)	7.2 (4.5-10.0)	6.2 (5.5-7.0)	
B-1	4.0 (3.0-4.5)	3.4 (2.0-4.5)			
Q-3 (31 mg/kg)		3.9 (2.0-5.0)	4.3 (3.0-10.0)	2.7 (2.0-4.0)	3.8 (3.0-5.0)
Q-4 (29 ")	6.2 (5.5-7.0)	5.8 (5.0-7.0)	4.6 (0.0-6.5)	5.5 (5.0-6.0)	6.0 (5.0-7.0)
Controls					
Q-2	3.2 (3.0-5.0)	3.6 (3.0-5.0)			
Q-1	2.9 (2.5-3.0)	3.0 (3.0-3.0)			

The numbers given in the columns under Period 1, Period 2, Period 3, etc., are the averages usually of 5 or 6 two-minute periods for a 20-minute duration. The figures in parentheses give the range of peristaltic activity for that period. Between Period 2 and Period 3, the nembutal was injected and after a short delay the counts were again begun.

on the influence of renal sympathectomy on kidney functions, but a study of ureteral peristalsis *per se* under various physiologic and experimental conditions was not carried out by these authors.

Results. The responses of the ureters in 2 experiments are plotted in Fig. 1. Experiment S-8 is a control experiment on an unanesthetized dog and shows the relative constancy of ureteral peristalsis over 100-minute period. Experiment S-7 shows the effect of a single rapid intravenous injection of pentobarbital sodium (nembutal). Peristaltic activity was reduced from an average of 5.6 spurts per minute to 1.1, followed by a gradual recovery.

The results of 11 experiments with varying

doses of intravenous nembutal and eight control experiments are summarized in Table I. The 19 experiments were performed on 3 dogs.

Conclusions. Observations of ureteral peristalsis can be made on unoperated and unanesthetized dogs and conclusions drawn as to the activity of the ureters. Although the series is small, results suggest that nembutal inhibits ureteral peristaltic activity. The mechanism of this inhibition may be through the influence of the anesthetic directly on the ureter; or the anesthetic may affect the urine production and thus the volume of urine passing down the ureter, since variations in urine volume will affect peristalsis of the ureters.

obtained by this method is rather hazy and cannot be easily compared with the tissue section. These disadvantages are obviated by coating the section with fluid photographic emulsion and staining it after development.⁵ A similar modification consists of mounting the sections directly on photographic plates.⁶ These techniques, however, resulted in erratic staining and some dissolution of silver granules in the staining reagents. The method, was, therefore, modified to permit staining of the section before application of the emulsion. This procedure, described below, results in an autographic image which may be observed under the high powers of the microscope (Fig. 1 and 2).

Technical Details. The thyroids of animals treated with radioactive iodine are fixed in Bouin's fluid or neutral formalin; mercury-containing fluids, such as Susa or Helly, cannot be used. The tissues are embedded in paraffin, sectioned at 3 to 5 micra and stained with either hematoxylin and eosin or Masson's trichrome. The stained sections, dehydrated through graded alcohols, are left for 1 minute in a 1% celloidin solution and dried. In the case of the trichrome stain, however, the slides should be quickly dipped into 1% celloidin for a few seconds, dried and dipped again. All celloidin-coated sections are then dried for at least 6 hours in order to harden the celloidin coating and thus protect the stains from the action of developer and fixer.

Subsequent operations are carried out in the darkroom at 2 to 3 feet from a No. 1 Wratten Safelight. The Kodak medium lantern slide plates used for the preparation of the coating emulsion are sampled for freedom from fog. The plates are soaked in distilled water for 10 minutes at $19^{\circ} \pm 1^{\circ}\text{C}$. The softened emulsion is then scraped into a 50 cc beaker, using the edge of another plate as a

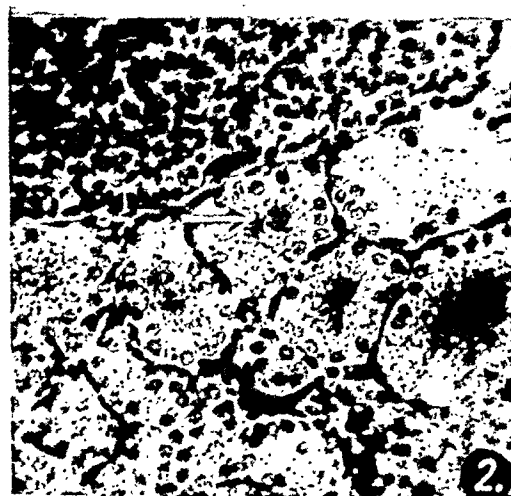
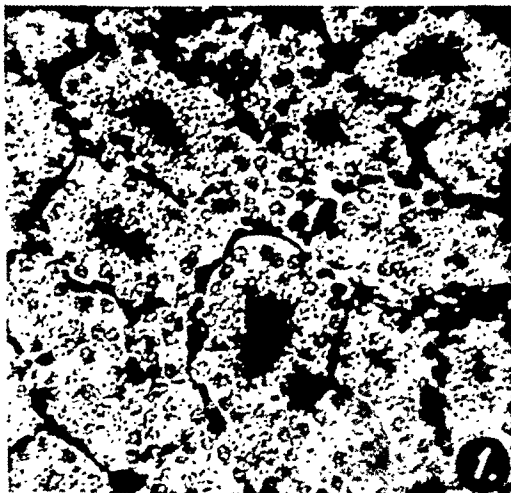


FIG. 1.

Section of thyroid of rat given radioactive iodide. The dark granules of silver, indicative of the presence of radioactivity, are seen in greatest density over the colloid contained in the thyroid follicles. Note that the thyroid epithelium shows a reaction only in the parts of the cells close to the colloid (as a result of the diffusion of the colloid image). The rest of the cell shows only background fog. The black outlines of the follicles represent the deeply staining red blood cells in the vessels. (H. & E., green filter, $\times 250$.)

FIG. 2.

Section of parathyroid (top) and thyroid of rat treated as above. Only a slight background fog is seen in the parathyroid. Thyroid reacts as in Fig. 1. The central arrow indicates a follicle where the colloid may be easily seen through the silver granules. (H. & E., green filter, $\times 250$.)

⁵ Hamilton, J. G., Soley, M. H., and Eberhart, K. B., *Univ. Calif. Publ. Pharmacol.*, 1940, **1**, 339.

⁶ Leblond, C. P., *J. Anat.*, 1943, **77**, 149.

⁷ Leblond, C. P., *Stain Techn.*, 1943, **18**, 159.

⁸ Belanger, L. P., and Leblond, C. P., *Endocrinology*, 1946, **49**, 8.

⁹ Evans, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 312.

infection, showing abortive fevers of one to 2 days duration, in contrast with the afebrile course of the animals treated with the anti-serum. The serological analysis of these "normal" sera gave evidence of lysins against sheep cells with titers of 1:20 or 1:40, and in some cases as high as 1:200. This has been recorded by other workers and attributed either to constitutional genetic factors (Witebsky and Neter⁶), or to past infections of heterogeneous origin (Hyde⁷). Attempts to investigate the possible role of the hemolysins by their absorption from both normal and immune sera with boiled sheep cells do not suggest correlation of heterophile antibodies with the observed protective action.

Experiments with guinea pig antiserum No. 91 also showed protective qualities when challenged with the highly virulent spotted fever, namely, abortive fever reactions, or complete absence of fever were observed. No greater protection was afforded by the globulin fraction of antiguinea pig serum (Complement fixation titer 1:300; hemolysin titer 1:1250) used in dilution 1:10. Slight elevations of body temperatures not exceeding 40°C for 1-2 days were recorded in 5 guinea pigs of the 10 used in the series. The temperatures of the other 5 animals remained normal over the 18 days observation. On the contrary,

the 3 untreated controls developed typical spotted fever. As observed in the typhus series, the course of spotted fever was occasionally attenuated by certain "normal" rabbit sera.

Conclusions. Antiginea pig rabbit immune sera, when injected intradermally into guinea pigs inhibited the clinical manifestations of typhus and spotted fever after the infective material was inoculated at the site of the serum administration. Highly protective properties against typhus and spotted fever, previously absent in the serum of untreated rabbits, were demonstrated in the antisera of the same animals after their immunization with guinea pig spleen and bone marrow. However, an attenuation of typhus and spotted fevers occurred when serum of certain "normal" rabbits was used. The presence of Forssman antibodies in the rabbit immune sera and of sheep cell lysins in the "normal" sera seems of no significance. Among the factors involved in the observed phenomena, the barrier effect of the skin and its modified permeability are probably decisive. The role of the spreading factor in the invasiveness of rickettsiae is under systematic experimental study.

The authors make no suggestions as to the possibilities of utilizing the methods and results described as a practical application for protection against or treatment of typhus or spotted fever.

⁶ Witebsky, E., and Neter, E., *J. Exp. Med.*, 1935, **61**, 489.

⁷ Hyde, R. R., *Am. J. Hyg.*, 1928, **8**, 205.

16209

Autographic Localization of Radio-Iodine in Stained Sections of Thyroid Gland by Coating with Photographic Emulsion.*

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Radioactive materials emit radiations which have the same effect as light on a photographic

* This work was supported by a grant from the National Research Council of Canada.

¹ Gross, J., and Leblond, C. P., *Canad. Med. Assn. J.*, 1947, **57**, 102.

emulsion. This property has been used to locate radio-elements in histological sections of tissues, and especially radio-iodine in the thyroid gland.¹ The radioactive section is usually placed in contact with a photographic film or plate.^{2,3,4} The image or "autograph"

Persistence of the Parabasal Body in a *p*-Rosaniline Resistant Strain of *Trypanosoma brucei*.

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It has been known for a long time (Werbitzki¹ that certain anti-trypanosomal drugs, *c. g.*, Pyronines, Acridines, Triphenylmethane dyestuffs, elicit a very definite response of the parabasal body of trypanosomes to the effect that this structure can no longer be demonstrated by the usual staining methods such as Wright's, Romanowsky's and Giemsa's stain. It is, moreover, known that trypanosome strains rendered resistant to these drugs may retain the lack of the parabasal body for many years. It was, therefore, surprising to note that in a strain of *T. brucei* which was made resistant to *p*-rosaniline hydrochloride the parabasal body persisted after the parasites had reached maximal resistance to this dyestuff. This rather unique observation seems interesting enough to justify a short description of the preparation and the properties of this drug resistant strain.

The strain of *T. brucei* was kindly given to us by Dr. M. Soule. According to a recent paper by Merchant² and a personal communication by Dr. Soule the strain was derived from an original isolation by Bruce (1896) and was kept in guinea pig passages for about 40 years. We became interested in these trypanosomes when it was observed³ that the organisms possessed a higher sensitivity towards *p*-rosaniline hydrochloride. It is well known that this triphenylmethane dyestuff is of very low activity. Most of the commonly used laboratory strains of *T. equiperdum*,⁴ *T. brucei*, *T. rhodesiense* require a subcutaneous treatment with the maximal tolerated dose

(50 mg/kg) or an oral administration of 250 to 500 mg/kg in order to clear the peripheral blood for a period of 3 to 6 days. No permanent cure of the infection can be obtained at least not with a single administration of the dyestuff. An infection of mice with the present *T. brucei* strain responded with permanent disappearance of trypanosomes to a single subcutaneous dose of 50 mg/kg or a single oral dose of 250 mg/kg. Smaller doses down to 10 mg/kg subcutaneously or 100 mg/kg orally cleared the peripheral blood for a 4-6 days period after which relapses occurred. Only doses of 5 mg/kg and 10 mg/kg given by the subcutaneous and oral route respectively failed to show any significant activity.

Drug resistance of this strain towards *p*-rosaniline hydrochloride was obtained by the so-called "short-passage method" described in an earlier paper.⁴ Starting with an oral dose of 10 mg/kg the strain became resistant to 500 mg/kg *per os* after 25 to 30 passages. The treatment with this dose of *p*-rosaniline was continued to a total of 47 passages in order to consolidate the fastness. The strain was eventually resistant to 500-1000 mg/kg *per os*. The dose of 1000 mg/kg which is close to the maximal tolerated dose still produced occasionally a more or less marked decrease in the trypanosome content of the peripheral blood, but never cured the animals.

Early in the process of rendering the strain resistant it was noted that the parabasal body persisted in the parasites. Despite the continued administration of *p*-rosaniline and the corresponding increase of resistance towards the dyestuff the parabasal body retained its tinctorial affinity and was easily demonstrated in smears from Giemsa's stain. This is shown in Fig. 1.

This persistence of the parabasal body in *p*-rosaniline-resistant trypanosomes has to our

¹ Werbitzki, F. W., *Centralbl. f. Bakteriol.*, 1910 I Orig., 1910, 53, 303.

² Merchant, D. J., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 391.

³ Ziering, A., and Buck, M., *E. Ch. Barch.*, Jubilee Volume, Basle, 1946, p. 378.

⁴ Schnitzer, R. J., Lafferty, L. C., and Buck, M., *J. Immunol.*, 1946, 54, 47.

scraper.[†] One lantern slide plate yields sufficient fluid emulsion to coat 3 or 4 microscope slides. The beaker is then placed in a water bath kept between 38° and 39°C. After 15 minutes in this bath, the emulsion becomes sufficiently liquid for application. A uniform emulsion coating is obtained through the use of a leveling table heated at one end to about 38°C and left at room temperature at the other end. This table was made of a plate glass top, with a heating element under one end, adjusted to give the required temperature. The whole is set on leveling screws and made horizontal with the aid of a spirit level.

The stained slides, after being warmed on the leveling table, are taken in one hand. Four to 8 drops of emulsion are applied with a medicine dropper held in the other hand. The air must be expelled from the dropper before dipping it into the emulsion, in order to prevent the occurrence of bubbles. About 2 drops of emulsion are applied per square inch of slide covered. The drops are spread evenly and quickly with a camel's hair brush. The slide is then rotated from side to side along its long axis to make the emulsion flow gently from edge to edge and thus obtain an even film. This may be seen by the red light reflected from the safelight on the surface of the emulsion.

The slides are returned to the warm side of the leveling table for 30 to 60 seconds and then gently slid to the cool side of the plate. The emulsion gels in about 15 minutes. The slides are then stored at 0° to +2°C in light-tight containers kept dry with P₂O₅. Test slides are developed at various time intervals after coating to determine the proper length of exposure. Development is in Kodak D-72 for 2 minutes at 19° ± 1°C with fixation in acid fixer for 10 minutes. The slides are then washed for 20 minutes, the water being kept below 20°C to prevent buckling and peeling of the emulsion. After passage through alcohol and xylol the sections are mounted in

balsam. Five minutes in each bath is necessary for complete dehydration. After placing the coverslips on the sections, these should be left to dry at room temperature. Throughout development and mounting, slides should be kept horizontal.

Discussion. A satisfactory autographic method should permit the visualization of histological details through the silver granules in the gelatin emulsion and thus enable a precise localization of these granules with reference to the stained structures in the section.

This requires the reduction of background fog to a minimum. Normally, unexposed lantern slides will show a certain amount of fog which increases with subsequent manipulations. This increase is avoided by careful control of temperature, minimum safe-light exposure, and storage at 0° to 2°C. In autographs with very little fog, it becomes possible to identify even minute accumulations of silver granules as resulting from the action of the radioactivity. Under these conditions, the length of exposure can be shortened; and the rather small reactions thus obtained make it easier to see the stained structures through the silver granules at the sites of radioactivity.

The photographic reaction due to the β -rays of I¹³¹ is maximal in the zone located immediately above a site of radioactivity and decreases as the square of the distance from this point. When such a reacting zone is examined through the microscope it appears circular; extending far from the source with prolonged exposure, but being more closely limited to it with controlled exposure. Indeed, with a short exposure, it is possible to limit the image to a zone almost directly above the emitter and thus obtain a nearly cytological localization (Fig. 1 and 2).

Summary. The autographic technic has been modified to permit staining of histological sections before coating with photographic emulsion. With this method, the diffusion of the image is sufficiently decreased to permit examination of the autograph under the high power of the microscope.

[†] Occasional batches of lantern slides are unserviceable as the emulsion does not soften in water and remains stringy and elastic.

TABLE I.
Sensitivity of the Normal and the *p*-Rosaniline Fast Strain of *T. brucei* Toward Different Anti-Trypanosomal Agents.

Compound	Route	Minimal active dose (mg/kg)	
		Normal strain	Resistant strain
Neo-arsphenamine	subcut.	12.5	12.5
Mapharsen	"	1.0	1.0
Acridlavine	"	5.0	5.0
Emetic tartar	"	5.0	5.0
6,6'-ureylene-bis (4-amino-2-methyl-quinoline)	"	62.5*	62.5*
<i>p</i> -Rosaniline HCl	"	10.0	> 50.0
	<i>per os</i>	100.0	> 500-1000.0

* Identical with the curative dose.

p-rosaniline-fast strains of *T. equiperdum** (PR I, PR III) which also lacked the parabasal body no longer responded to subcutaneous treatment with 500 mg of the quinoline derivative per kg, while a tenth of this dose (50 mg/kg) cured the infection with the parent strain.

These observations indicate that an atypical *p*-rosaniline-fast strain of *T. brucei* was obtained. It was characterized by the persistence of the parabasal body and the therapeutic sensitivity to 6,6'-ureylene bis (4-amino-2-methylquinoline). *p*-Rosaniline-resistant modifications of *T. equiperdum* exhibited as a rule the loss of both these properties. The question of the mechanism by which these

2 phenomena, namely, presence or absence of the parabasal body and sensitivity towards 6,6'-ureylene bis [4-amino-2-methyl quinoline] are related or whether they are related at all cannot be decided on the basis of our present experience. We are also not in a position to state definitely that other strains of *T. brucei* might give a similar response to *p*-rosaniline or even that the same strain of *T. brucei* will always react in the same atypical way to attempts of rendering it *p*-rosaniline-fast.

Summary. A *p*-rosaniline-resistant modification of *T. brucei* is described in which—contrary to the experience with other trypanosome strains—the parabasal body persisted. The strain also failed to show the overlapping resistance to 6,6'-ureylene [4-amino-2-methyl-quinoline] which could be demonstrated in *p*-rosaniline-fast strains of *T. equiperdum*.

* The experiments with *T. equiperdum* were carried out by Schmietzer in the Connaught Medical Research Laboratories of the University of Toronto.

16211

Stability of Natural Progesterone.*

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In 1936 approximately one gram of crystalline progesterone was isolated from sows'

ovaries for use in the study of some of the biological properties of the hormone.¹ The major portion of this lot was used in the study of various aspects of the hormonal control of

* The authors wish to thank Miss Lila F. Knudsen, of the Food and Drug Administration, Washington, D.C., for her assistance in the statistical analysis of the data.

¹ Allen, W. M., and Goetsch, Carl, *J. Biol. Chem.*, 1936, **116**, 653.

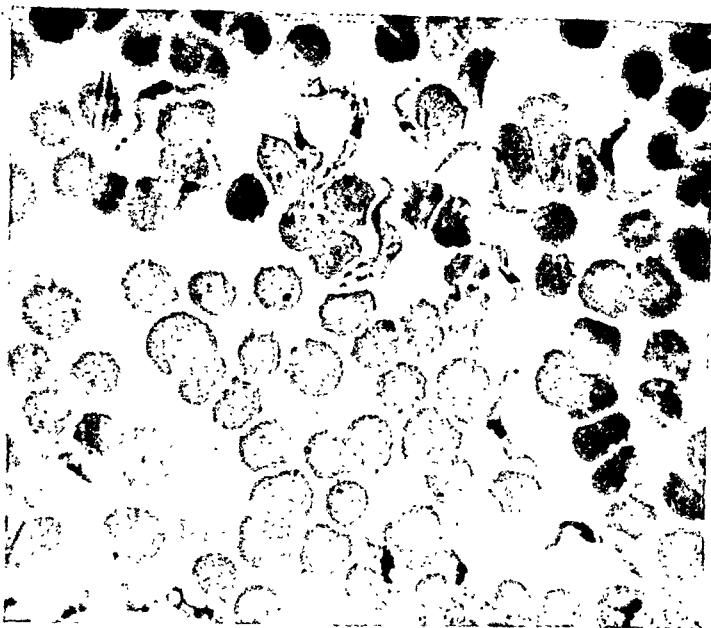


Fig. 1.

Blood of a mouse infected with the *p*-rosaniline resistant strain of *T. brucei*. (47th passage.) Giemsa's stain. Objective apochromate HE 90, compens. eye piece 10 \times . (Photomicrograph by L. Hodax.)

knowledge not been described before. In a number of trypanosome strains rendered resistant to the dyestuff in our laboratory a similar phenomenon was never observed. In particular, of 9 *p*-rosaniline-fast strains of *T. equiperdum* which were prepared in mice and rats and studied during recent years none failed to show the absence of the parabasal body in 80-100% of the individual parasites.

The unusual observation in the resistant strain of *T. brucei* made it desirable to examine the properties of this strain more closely and particularly to investigate its response to different anti-trypanosomal agents.

The results of these specificity tests of the resistant strain are given in Table I.

Mice infected with the parent strain and the *p*-rosaniline-fast trypanosomes were treated with a group of different chemotherapeutic agents of known activity such as 3,3'-4,4'-dihydroxy arsono-benzene-*N*-methan-*al* sulfoxylate (Neoarsphenamine), 3-amino-4-hydroxyphenyl arsineoxide hydrochloride (Mapharsen), potassium antimonyl tartrate

(Emetic tartar), 2,8-diaminoacridinium-10-methochloride (Acriflavin), and a quinoline compound, 6,6'-ureylene bis[4-amino-2-methyl quinoline].

From the data of the minimal active doses, as given in the table, it is evident that the resistance was specific for *p*-rosaniline; the arsenicals, the antimony compound, and the acridine dyestuff showed the same activity on the resistant as on the parent strain. That is characteristic for *p*-rosaniline-fast trypanosome strains.

The observation that the quinoline compound was also active in the infection with the *p*-rosaniline-fast strain was surprising. This compound is a member of a large series of anti-trypanosomal agents⁵ and according to previous experience (Schnitzer, unpublished data) proved to be active in experimental infections with normal trypanosomes but without effect on the *p*-rosaniline-resistant modifications of these strains. For instance: Two

⁵ Jensch, H., *Angewandte Chemie*, 1937, 50, 891.

TABLE I.
Original Bioassay of Progesterone in 1934 (Corner-Allen Method).
Endometrial Proliferation.

Prep. No.*	Dose, mg	1934	1946†	R	Log dose $\times 10$	θ
Alpha Progesterone.						
1	1.77‡	4+	4+	.723	1.248	58.3
1	1.33	4+	3+	.816	1.123	64.6
2	1.22	4+	3+	.675	1.086	55.2
3	0.91	3+	3+	.502	.959	45.1
2	0.90	3+	3+	.696	.954	56.6
1	0.88	2+	2+	.544	.944	47.5
2	0.61	1+	1+	.292	.785	32.7
3	0.60	1+	1+	.218	.778	27.9
1	0.44	1+	2+	.313	.643	34.0
Beta Progesterone.						
2	1.47	4+	4+	.802	1.802	63.6
3	1.28	4+	4+	.784	1.107	62.3
1	1.17	3+	4+	.767	1.068	61.1
2	1.05	2+	2+	.582	1.021	49.7
3	0.94	2+	1+	.402	.973	39.3
1	0.70	2+	3+	.588	.845	50.1
2	0.63	1+	1+	.304	.799	33.4
3	0.63	1+	2+	.357	.799	36.7

* Preparation numbers same as the number from Table I, *J. Biol. Chem.*, 1934, **107**, 321.

† Re-evaluation of the proliferation of the original sections.

‡ Data from animals receiving 1.77 and 1.33 mg of alpha and 1.47 of beta were excluded in the determination of the regression lines.

knowledge of the original interpretations. The estimates of the degree of proliferation of the endometrium were virtually the same as originally.

The bioassay of the samples of alpha and beta progesterone after storage for ten years is recorded in Table II. Several animals were used at each of several levels of dosage, spread over the critical range, to find out how much variation in response is to be expected.

Comparison of the data in Tables I and II shows, first of all, that the quantities necessary to produce full proliferation, and the quantities which produce little or no proliferation, are virtually the same. This indicates beyond doubt that there has been no great change in activity during the interim. Secondly, the variability of response in the critical range of dosage seems to be about the same.

As yet there is no generally accepted method for comparing the activity of compounds having progestational activity with the activity of the international standard of progesterone. Inspection of Tables I and II certainly indicates that the preparations obtained in 1936, and assayed in 1946, have approximately the same activity as the original preparations obtained and assayed in 1934. There is every reason for believing that the results should be

similar, since the physical characteristics remained essentially unchanged during the storage period. More detailed comparison of the results of the bioassays, therefore, is justified as it affords in a sense an indication of the reliability of bioassays of identical compounds at widely separated periods of time, even though the results can only be compared with each other and not with concurrent assays of the international standard.

The best approximation of a linear relationship between the dose administered and the response of the endometrium (R —obtained by the planimeter) was secured when the dose was transformed to log dose and the angular transformation[†] was used on the response. The data for each of the four preparations were subjected to the above transformation and the dosage response curves fitted by the method of least squares. Only tests falling in the critical range (0.4–1.2 mg) were used. The results of this form of analysis of the data are recorded in Table III. The slopes are not very steep, but in all cases they are statistically significant, since in each case the slope

† Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural, and Medical Research*, Table XII, p. 42, Oliver and Boyd, Edinburgh, 1938.

pregnancy in rabbits. During that study no bioassays of the hormone were made to specifically test its stability. The compound was assumed to be stable since the physical characteristics of the crystals did not appear to change with the lapse of time. Small portions of the preparations obtained in 1936, however, were kept for bioassay at a later date to determine their stability. These samples of alpha and beta progesterone have now been assayed and have been found to be as active today, after storage for 10 years, as the first preparations obtained by Wintersteiner and Allen in 1934.²

The preparations recently subjected to bioassay were stored during the past decade in small, unsealed, glass-stoppered, vials. The vials were in turn subjected to the temperate environment of a desk drawer without any further attention until the samples were removed for the present experiment.

The melting points of the compound, after 10 years, were essentially the same as originally. The present sample of alpha progesterone was made up of the unused portions of several lots, the original melting points of which ranged between 126° and 129°C. The melting point obtained recently was 123°-124°C. The present sample of beta progesterone melted at 120°-121°C; the original lots from which the present small sample was obtained had melting points ranging from 118°-121°C.

Both the alpha and beta forms were bioassayed according to a slight modification of the original Corner-Allen method.^{3,4} Our modified method differed from the original in that ovulation was induced in the rabbits used for the tests by employing commercial chorionic gonadotrophin rather than by mating the animals with the male. Ovulation was obtained by injecting intravenously a freshly prepared aqueous solution of powdered, dry, human chorionic gonadotrophin equivalent to approximately 20 international units. Laparotomy

was performed 18 to 24 hours after the administration of the gonadotrophin. At the time of the operation, the ovaries were inspected and if ovulation had occurred, both ovaries were removed and the distal third of one uterine horn resected to serve as a histological control. Beginning on the day of operation and on each day thereafter for a total of 5 days, one-fifth of the total dose of progesterone was administered subcutaneously in 0.2 cc of sesame oil. Twenty-four hours after the last dose, the animals were sacrificed and the uteri removed and fixed in formalin. Transverse sections of the uterine horns were then prepared and stained with hematoxylin and eosin in the usual manner.

The degree of endometrial proliferation was determined by two methods: (a) by comparison with illustrations already published⁴ and (b) by measuring the amount of proliferation with a planimeter. The planimetric measurements were made from tracings of the projected image of the section drawn on white paper in the following manner. Three circumscribing lines are drawn in different colors to locate the areas for measurement. Since degree of proliferation is determined as a ratio, the degree of magnification does not need to be kept constant. First, a line (M) is drawn which separates the myometrium from the endometrium. Next, a line is drawn which outlines the outermost aspect of the glands (S). This line divides the endometrium into an outer, unproliferated, non-glandular area and an inner glandular area. This line coincides with the line designated as (M) in some regions of the well proliferated uteri. Finally, a line (L) is drawn separating the lumen from the glands. These lines are separately traced with the planimeter and the circumscribed areas determined. The portion of the endometrium which has become glandular (R) is given by the formula:

$$R \text{ (response)} = \frac{S - L}{M - L} = \frac{\text{area of glands}}{\text{area of endometrium}}$$

For the purpose of comparison, Table I is compiled from bioassay data obtained in 1934 using the original Corner-Allen method. The sections were re-evaluated without exact

² Wintersteiner, O., and Allen, W. M., *J. Biol. Chem.*, 1934, **107**, 321.

³ Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, 1929, **88**, 326.

⁴ Allen, W. M., *Am. J. Physiol.*, 1930, **92**, 174.

thus obtained does not differ significantly from 100%.

Summary. Natural progesterone isolated from pigs' ovaries in 1936 shows no loss of activity after storage for 10 years in glass-stop-

pered vials at room temperature. Essentially similar methods of bioassay at widely separated intervals of time give dosage response curves that show no statistically significant differences.

16212

Relation of Complement to Blood Coagulation.

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Evidence has been presented both for and against the participation of complement in the coagulation of the blood. Wadsworth, Maltaner and Maltaner¹ favored the affirmative view because many substances were found to have both anticoagulant and anticomplementary actions. Maltaner² reported a close correlation between thromboplastic and complement-fixing power of tissue extracts and sera. On the other hand, Wilander³ and Ecker and Pillemer⁴ found great quantitative differences between the relative anticoagulant and anticomplementary properties of a number of substances. Quick⁵ showed that complement as a whole is not the same as prothrombin. However, much recent work points to the existence of an additional component of the coagulation system, concerned with the conversion of prothrombin to thrombin. We⁶ have already quoted much of this work but

should mention in addition the contributions of Ware, Guest, and Seegers^{7,8} and the extensive and convincing investigations of Owren.⁹ A study of the role that complement plays in the conversion of prothrombin to thrombin appears timely.

Methods. Human plasma was freed of complement activity in 3 ways: by aging and by treatment with zymin and with ammonia. After aging for 2 to 3 months at icebox temperature plasma had no complement activity. Zymin powder was prepared from yeast essentially as described by Ecker, Jones, and Kuehn.¹⁰ Two hundred and fifty milligrams of zymin were mixed with 1 cc of oxalated plasma and 1 cc of imidazole buffer at pH 7.2. Ordinarily serum is incubated with zymin for one hour at 37°C in order to inactivate complement, and smaller quantities than used by us suffice. We found that if the plasma was centrifuged immediately after thorough mixing with zymin, most but not all of the complement activity had disappeared by the time the supernatant was tested, about 20 minutes after the addition of zymin. The remaining complement activity disappeared gradually over a period of one to 2 hours in the absence of zymin. This was the procedure followed in preparing the zymin plasma used in the experiments described subsequently. This

¹ Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

² Maltaner, Frank, *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 302.

³ Wilander, Olof, *Skandinav. Arch. f. Physiol.*, 1938, **81** (Suppl. 15), 89 pp.

⁴ Ecker, E. E., and Pillemer, L., *J. Immunol.*, 1941, **40**, 73.

⁵ Quick, A. J., *J. Immunol.*, 1935, **20**, 87.

⁶ Mann, F. D., Hurn, Margaret, and Magath, T. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **60**, 33.

⁷ Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **100**, 41.

⁸ Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

⁹ Owren, P. A., *The Coagulation of Blood; Investigations on a New Clotting Factor*, Oslo, J. Chr. Gundersen, Boktrykkeri, 1947, 327 pp.

¹⁰ Ecker, E. E., Jones, C. B., and Kuehn, A. O., *J. Immunol.*, 1941, **40**, 81.

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TABLE II.
Bioassay of Progesterone After Storage for 10 Years (Modified Corner-Allen Method).
Endometrial Proliferation.

Dose, mg	Proliferation, Avg	R, Avg	Log dose $\times 10$	θ
Alpha Progesterone.				
1.6*	4+	.702	1.204	56.9
1.4	4+	.536	1.146	47.1
1.4	4+	.651	1.146	53.8
1.4	4+	.837	1.146	66.2
1.2	3.5+	.741	1.079	59.4
1.2	3.5+	.708	1.079	57.3
1.2	3.5+	.805	1.079	63.8
1.0	2.5+	.650	1.000	53.7
1.0	2.5+	.646	1.000	53.5
1.0	3.5+	.664	1.000	54.5
.8	2.5+	.632	.903	52.6
.8	3.5+	.620	.903	51.9
.8	2+	.581	.903	49.7
.8	2+	.508	.903	45.5
.6	2+	.457	.778	42.5
.6	+	.406	.778	39.6
.6	2+	.461	.778	42.8
.6	+	.373	.778	37.7
.4	0	.182	.602	25.2
.4	+	.450	.602	42.1
.4	+	.476	.602	43.7
.4	+	.433	.602	41.2
.2	0	.143	.301	22.2
.2	0	.193	.301	26.0
.2	0	.158	.301	23.4
Beta Progesterone.				
1.2	3+	.691	1.079	56.3
1.0	3+	.614	1.000	51.6
1.0	3.5+	.714	1.000	57.7
.8	1+	.330	.903	35.1
.8	4+	.761	.903	60.8
.8	2+	.541	.903	47.4
.8	2+	.488	.903	44.3
.6	3+	.632	.778	52.6
.6	5+	.273	.778	31.5
.4	+	.438	.602	41.5
.4	0	.026	.602	9.2

* Data from animals receiving 1.6, 1.4, and 0.2 mg were excluded in the determination of the regression lines.

TABLE III.
Analysis of the Dosage Response Curves (log dose — θ response).

Preparation	No. of animals	Intercept of regression line	Standard error of estimate	Slope	Standard error of slope
1934 Alpha	7	50.5	6.02	64.5	15.2
1934 Beta	7	51.6	6.54	73.4	19.1
1946 Alpha	18	54.4	4.69	46.6	6.4
1946 Beta	11	53.8	10.67	66.9	20.7

is more than 3 times its standard error. Likewise the values for the intercept at a dose of 1.0 mg show very little variation. The data indicate, therefore, that the dosage response curves from the data of 1934 do not differ significantly from those obtained from assay

in 1946 of preparations that were 10 years old. Likewise there is no significant difference between the curves for alpha and beta progesterone. Using a biological assay procedure with the 1934 data labeled "standard" and the 1946 data labeled "unknown," the potency

TABLE II.

Two-Stage Assays: Dilution of Various Plasmas Is That in the 2 cc of Incubation Mixture Containing Calcium and Thromboplastin. Units of Prothrombin Are Calculated in Terms of Original Plasma or Total Plasma When Aged Plasma Is Included. Amount of Serum Is That Added to the 2 cc Incubation Mixture. The Very Low Yields of Thrombin Give Clotting Times Too High for Accurate Determination.

Plasmas and dilutions		Serum, cc	Yield of thrombin, units per cc of plasma
Untreated	1:100	0	490
None		0.1	< 5
Zymin	1:40	0	Slight trace
"	1:40	0.1	280
NH ₃	1:40	0	< 20
"	1:40	0.1	290
Zymin	1:80 + NH ₃ 1:80	0	320
Aged	1:40	0	< 20
"	1:80	0.1	300
"	1:80 + Zymin 1:80	0	300
"	1:80 + NH ₃ 1:80	0	< 20

N.B. Units of prothrombin are determined in terms of yield of units of thrombin.

TABLE III.

Tests for Complement Activity: 1 cc Sensitized Sheep Cells Added to Each of the Following.

Components present	Hemolysis
.1 cc serum + 0.1 cc saline	Complete
.1 " untreated plasma + 0.1 cc saline	"
.2 cc zymin plasma	None
.2 " NH ₃ "	"
.2 " aged "	"
.1 " zymin "	"
+ 0.1 cc NH ₃ plasma	Partial
.1 cc zymin plasma	"
+ 0.1 cc aged plasma	"

bin while leaving abundant prothrombin which is highly reactive in the presence of the proper conversion factors, makes it somewhat doubtful whether any method of assay yet devised measures a single substance, pro-

thrombin. The foregoing experiments point to a complex rather than a simple prothrombin conversion factor.

The inactivations of the third and fourth components of complement by zymin and by ammonia respectively are regarded as fairly specific, being in fact the bases for definition of these components. To regard the blood coagulation system and complement as closely related appears to be the point of view most likely to direct efforts toward increasing knowledge of both.

Summary. Inactivation of the complement of plasma by aging or by treatment with zymin or with ammonia blocks conversion of prothrombin to thrombin while leaving ample reactive prothrombin.

16213

Cultivation of Toxoplasma in Embryonated Egg. An Antigen Derived from Chorioallantoic Membrane.

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Neutralization¹ and complement-fixation² tests have been used for establishing the diag-

¹ Sabin, A. B., and Ruchman, I., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 1.

nosis of toxoplasmosis in man; however, neither method is entirely satisfactory. The

² Warren, J., and Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 11.

TABLE I.

One-stage Assays: 0.1 cc of Standard Rabbit Brain Thromboplastin and 0.1 cc of 0.025 M Calcium Chloride Added to Tubes Containing 0.1 cc of Each of the Following Pairs of Components (Total Volume 0.4 cc).

Components present	Complement	Clotting time, sec.
Untreated plasma and saline	Present	19
Zymin plasma and saline	Absent	202
" " " serum	Present	20
NH ₃ plasma and saline	Absent	190
" " " serum	Present	22
Zymin plasma and NH ₃ plasma	"	32
Aged plasma and saline	Absent	186
" " " serum	Present	21
" " " zymin plasma	"	28
" " " NH ₃ plasma	Absent	193

zymin-treated plasma, when mixed with four volumes of fresh oxalated plasma and incubated at 37°C, removed in several hours most of the complement activity of the added plasma. These observations, which suggest that the action of zymin is not one of simple adsorption, are being studied further. Ammonia-treated plasma was prepared by incubating the mixture of 1 cc oxalated plasma, 1 cc imidazole buffer and 0.2 cc of 1% ammonium hydroxide for one hour at 37°C. This mixture had a pH of 8.2 and approximately the minimal concentration of ammonia reported as necessary to inactivate the fourth component of complement in serum.¹¹ After the treatment with ammonia the plasma was devoid of complement activity. To test for complement 0.2 cc of treated plasma was added to 1 cc of a 1% suspension of sheep cells sensitized with 2 units of amboceptor and the mixture was incubated for one hour at 37°C. Serum was also inactivated by zymin and by ammonia by the same procedures used for plasma. Thrombin-forming activity was tested by a one-stage procedure using a system of 0.4 cc total volume⁶ and by a 2-stage procedure,¹² both exactly as previously described except that the plasma was not defibrinated in the 2-stage method.

Results. In all 3 types of complement-inactive plasma, marked loss of thrombin-forming ability was observed for both one-stage and 2-stage methods. The rapid,

marked increase in the one-stage prothrombin time on aging is, of course, well known: after prolonged aging, as in these experiments, the yield of thrombin with the 2-stage procedure is also very low. Striking decrease of the one-stage prothrombin time and increase of the 2-stage thrombin yield were obtained with all 3 types of treated plasma by use of 0.1 cc of serum in the 0.4 cc one-stage system and by addition of 0.1 cc of serum to the 2 cc of incubation mixture in the 2-stage procedure. The serum used (4 to 6 hours subsequent to spontaneous coagulation) did not clot fibrinogen and contained no fibrinogen and only very small amounts of prothrombin as shown by the 2-stage assay. Serum inactivated with zymin or ammonia did not restore thrombin-forming activity to similarly inactivated plasma. Mixtures of zymin plasma and aged plasma formed thrombin readily and had appreciable, although not normal, complement activity. Mixtures of aged plasma and ammonia plasma showed no restoration of thrombin formation and little or no complement activity. The addition of serum inactivated by heating at 56°C for 30 minutes partially restored complement activity to ammonia-treated plasma but did not restore thrombin-forming ability. An illustrative protocol (Tables I, II, and III, all data obtained with the same reagents on the same day) shows most of the effects observed. All of the foregoing experiments were easily reproducible.

Comment. The observation that plasma, by 3 different methods, may be almost completely deprived of its ability to form throm-

¹¹ Pillemer, L., Seifter, J., and Ecker, E. E., *J. Immunol.*, 1941, 40, 89.

¹² Hurn, Margaret, and Mann, F. D., *Am. J. Clin. Path. (Tech. Sect.)*, 1947, 17, 741.

TABLE I.
Distribution of Toxoplasma in Embryonic Tissues.

Egg passage No.	Days after infection	Intracerebral infectivity titer of tissue for mice				
		Emb. brain	Emb. torso	Allant. memb.	Allant. fl.	Yolk sac
1	7	10-4.4	10-4.5+	10-4.5+	—	10-4.0
2	7	10-2.6	10-3.5	10-3.2	—	10-1.5
3	8	10-4.4	10-3.5	—	10-1.8	10-2.5
3A	7	—	—	10-5.5	—	—
5	8	10-4.6	10-3.5	10-3.5	—	10-2.5
11	7	10-4.5	10-4.0	10-3.8	—	10-2.0

Embryos were 10 days old at time of inoculation and were incubated at 35°C after infection.

sults in a fatal infection. Growth of the parasite occurs equally well in embryonated eggs which are 6 to 12 days old when inoculated; embryos succumb between the seventh and tenth days after infection with practically all dying on the eighth day. Multiplication of the organism occurs equally well in eggs incubated at 35° or 37.5°C.

Toxoplasma are demonstrable microscopically in all tissues of infected embryos. Tissues and fluids of inoculated eggs have infective titers of $10^{-2.0}$ to $10^{-5.5}$ when tested in mice. However, as indicated in Table I, the highest concentrations of the parasites usually are found in the embryo and allantoic membrane.

The most striking pathological lesions in the infected embryonated eggs are large yellowish-gray nodules, scattered throughout the allantoic membrane (Fig. 1). These may be 2 mm in diameter and are frequently visible through the unbroken shell upon transillumination. The embryos themselves are deeply hemorrhagic and occasionally show nodular lesions in the viscera or the skin. The nodules on microscopic examination consist of a central area of necrosis surrounded by a zone of mononuclear leukocytes and contain many toxoplasma scattered throughout the necrotic and infiltrated portions of the lesion.

(b) *Complement-fixing Antigen.* Antigens prepared from infected chorioallantoic membrane were compared in complement-fixation tests with antigens derived by a similar procedure from infected rabbit and mouse brain tissues.² Antigens from all 3 sources had titers of 1/8 to 1/16 when tested with 2 units of antibody. The complement-fixing material in the chick embryo preparation had the characteristics of a specific soluble substance since

it was separable from the intact organism; data presented in Table II show that no diminution in activity resulted when chick embryo antigens were passed through a Seitz filter or were centrifuged at 14,000 r.p.m. in an angle machine for one hour. Moreover, the antigens are relatively stable; complement-fixing activity was not reduced by lyophilization nor by storage at -20°C for 3 months.

(c) *Preliminary Evaluation of Complement-fixation for Diagnosis of Human Toxoplasmosis.* A number of stored frozen samples of serum were available which had been submitted from adults and children suspected of having toxoplasmosis, all of which had been previously tested for neutralizing antibody. In addition, control sera obtained from "normal" and from syphilitic adults were examined. The above sera were tested for complement-fixing antibodies employing antigen prepared from chorioallantoic membrane. Four of the 11 "normal" sera and 3 of the 14 sera with positive Wassermann reactions fixed comple-



FIG. 1.
Chorioallantoic membrane infected with toxoplasma.

former requires the use of living parasites and test animals, whereas the rabbit-brain antigen, generally used in the latter, gives non-specific reactions under certain conditions. The present report describes the successful cultivation of toxoplasma parasites in embryonated eggs together with a method for preparing complement-fixing antigen from infected chick embryo tissue. Preliminary diagnostic data obtained using this antigen in tests with sera of patients are included.

Materials and Methods. (a) *Strain of Toxoplasma and Its Maintenance in Embryonated Eggs.* The RH strain of toxoplasma of human origin³ was employed in the present studies. Infected mouse brain material was inoculated into embryonated eggs and the agent subsequently was maintained by serial passage in the avian host. For this purpose the brains of those embryos which appeared moribund 7 to 8 days after inoculation were removed, and a 10% suspension of tissue, partially clarified by light centrifugation, was injected in 0.1 cc amounts into the chorioallantoic sacs of normal embryos. Impression films, prepared from the brain tissue of selected embryos, were stained by Giemsa's method and the presence of toxoplasma was verified before passage was performed.

(b) *Preparation of Complement-fixing Antigen.* Chorioallantoic membranes bearing macroscopic lesions were harvested from 12 or more eggs on the seventh day after inoculation. These were pooled, ground in a mortar with sterile alundum and a 10% suspension was prepared by adding physiological saline solution buffered at pH 7.4. This crude suspension was frozen and thawed 3 times and then clarified by centrifugation at 3,500 r.p.m. for 15 minutes in an angle machine. The supernatant fluid, after addition of sufficient merthiolate to bring the concentration to 1:10,000, constituted the antigen. This was generally stored at -20°C until used. Normal 16-day-old chick embryos were similarly treated to prepare an antigen for use as control material.

(c) *Specific Immune Sera.* Immune sera were obtained from rhesus monkeys and from guinea pigs convalescent from infection with

toxoplasma. Monkeys were infected by the subcutaneous inoculation of 0.1 cc of mouse brain suspension containing approximately 10,000 to 100,000 minimal lethal doses of the organism as estimated by intracerebral titration in mice. Guinea pigs were inoculated intraperitoneally with 100 to 1,000 minimal lethal doses of toxoplasma similarly estimated. Both monkeys and surviving guinea pigs were bled for immune serum approximately 30 days after inoculation.

Sera from a number of patients who had been suspected of having a toxoplasma infection were available for study. These sera had been stored at -20°C for from a few days up to 3 years.

(d) *Neutralization Tests.* The presence in serum of neutralizing antibodies against toxoplasma was determined by the method of Sabin and Ruchman.¹ In this procedure mixtures of serum and infectious mouse brain suspension are inoculated intracutaneously into rabbits and the resultant lesions are read on the fourth to seventh day.

(e) *Complement-fixation Tests.* Tests for complement-fixing antibodies were performed in the following manner: Tubes containing 0.25 cc amounts of the appropriate dilutions of the serum to be tested received 0.25 cc of antigen (4 units) and 0.5 cc of fresh diluted guinea pig serum containing 2 units of complement as determined by preliminary titration in the presence of four units of antigen. These were incubated overnight at 5°C . The hemolytic system, consisting of 0.5 cc of an equal mixture of a 3% suspension of washed sheep erythrocytes in saline and diluted amboceptor (2 units), was then added to the tubes. The mixtures were incubated at 37°C for a half hour and then read in the usual manner. In addition to the usual controls, each serum was tested with normal antigen employed at a dilution comparable to that of the toxoplasma antigen.*

Experimental. (a) *Cultivation.* The introduction of toxoplasma into the chorioallantoic, amniotic, or yolk sacs almost invariably re-

* All complement-fixation tests reported in this paper were performed by Mr. M. J. Snyder of this department to whom the authors wish to express their gratitude.

³ Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

TABLE III.
Positive Serological Reactions of Patients Having Clinical Evidence of Toxoplasmosis.

Patient	Clinical manifestations	Test	
		Neutralization*	Complement-fixation (Titer)
A	Fatal case of toxoplasmosis in infant	Strongly pos.	1:32
B	Infant with chorioretinitis, cerebral calcification and mental retardation	Positive	1:128
C	Healthy mother of infant A	Strongly pos.	1:128
D	Healthy mother of infant B	Positive	1:128
E	Healthy mother of infant with congenital cataracts	"	1:16
F	Adult with cutaneous nodules, chorio-retinitis and fever	"	1:128

* Neutralization by the test serum of a 1:100 dilution of toxoplasma-infected tissue when the mixture is injected intracutaneously in the rabbit is regarded as a positive reaction; neutralization of a 1:20 dilution as a strongly positive.

blood, and sternal marrow gave negative results. Because of these serological findings, the patient was tested for cutaneous reactivity to toxoplasma material. For this purpose, 1/100 and 1/1000 dilutions of standard complement-fixing antigen and a 1/1000 dilution of normal egg antigen prepared in a similar fashion were injected intracutaneously in 0.1 cc amounts. Although there was no immediate reaction at any of the sites, both of the areas receiving the toxoplasma antigen developed erythema and induration at 24 hours which persisted for several days; the lesion at the site of the 1/100 dilution was $1\frac{1}{2} \times 2 \times 0.1$ cm and at the 1/1000 dilution was $1 \times 1 \times 0.1$ cm. No reaction was elicited at the site of the injection of normal egg material.[†]

Summary. Inoculation of embryonated eggs with toxoplasma resulted in a generalized parasitic disease of this host with a fatal termination. The agent could be maintained by serial passage of embryo brain. Chorioallantoic membranes from infected eggs contained a stable, specific soluble antigen which fixed complement with toxoplasma immune animal serum. Human sera from both proven and suspected cases of toxoplasmosis which were known to contain neutralizing antibody against the parasite also fixed complement with chorioallantoic membrane antigen.

† These studies were performed in collaboration with Drs. A. J. Brennan and T. Brown, Mount Alto Hospital, Washington, D.C., and will be reported in detail elsewhere.

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Influence of Oxophenarsine on Hypoglycemic Action of Insulin.

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Noting the results obtained by Barron and Singer¹ in inhibiting the activity of certain enzymes with arsenoxides and their use of this reaction as a test of the essential SH groups

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¹ Barron, E. S. G., and Singer, T. P., *Science*, 1943, 97, 356.

of such enzymes, the author thought it of interest to incubate insulin under sterile conditions with the arsenoxide, oxophenarsine (3-amino-4-hydroxyphenyl arsenoxide hydrochloride). While enzyme workers assume that reaction is only with free SH groups, it seemed possible that a part of the arsenoxide might reduce the S-S linkages of insulin to -SH.

TABLE II
Complement-Fixation Reactions with Toxoplasma.
Toxoplasma chorioallantoic membrane antigen

Serum used	Serum dilution	Crude suspension				After desiccation and dehydration				Seitz filtrate				14,000 rpm/60'				Normal antigen Crude suspension
		1:2		1:4		1:8		1:16		1:2		1:4		1:8		1:16		
Toxoplasma immune guinea pig	1:25	4+	4+	4+	0	4+	4+	4+	0	4+	4+	+	0	4+	4+	4+	0	0
	1:50	4+	4+	4+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	3+	0	0
	1:100	4+	4+	+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	0	0	0
	1:200	4+	3+	0	0	4+	4+	0	0	+	2+	0	0	+	3+	+	0	0
	1:400	0	0	0	0	2+	3+	0	0	0	0	0	0	0	0	0	0	0
Normal guinea pig	1:25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

ment at dilutions of 1/4 to 1/8 with both toxoplasma and normal egg antigens. All of the "normal" sera which reacted in these tests, however, were obtained from individuals immunized with vaccines of egg origin, and fixation of complement by mixtures of normal egg antigen and sera from vaccinated or syphilitic persons is well known.^{4,5} By contrast, sera from 47 adults with clinical diagnoses of chorio-retinitis, uveitis, iritis, or visual impairment, all of unknown etiology, were examined. None of these sera had significant amounts of neutralizing or complement-fixing antibody of toxoplasmosis. Tests were also made on sera from 7 children, aged from 6 weeks to 2½ years, on whom a diagnosis of toxoplasmosis was entertained because of 2 or more of the following manifestations: chorio-retinitis, hydrocephalus, cerebral calcification, and mental retardation. Both complement-fixation and neutralization tests were negative with 5 sera and both were positive with the remaining two (infants A and B in Table III). The diagnosis of toxoplasmosis was proved in infant A by recovery of the parasites in inoculated animals and their microscopic demonstration in human autopsy material.

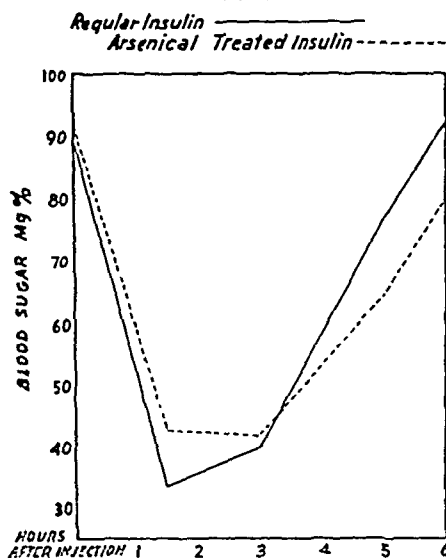
Sera from the healthy mothers of infants A and B both contained neutralizing and complement-fixing antibodies for toxoplasma (Table III). Both types of test were also positive on sera from a third mother who delivered a child with congenital cataracts; serum from the child was not available for study. Negative results were obtained in both types of test with sera from 2 families, mother and child, in which the infant was suspected of having toxoplasmosis on clinical grounds.

The serological results obtained in the case of a 30-year-old male with a peculiar syndrome are worthy of mention. This man (patient F in Table III) had a nodular dermatitis, chorio-retinitis, weakness, weight loss, eosinophilia and low grade fever, all of approximately 2 years duration. His serum gave a complement-fixing titer of 1/128 and a strongly positive neutralization test. Repeated attempts to recover toxoplasma from cutaneous lesions,

⁴ Wertman, K., *J. Lab. Clin. Med.*, 1945, **30**, 112.

⁵ Smadel, J. E., Warren, J., and Snyder, M. J., *J. Bact.*, 1947, **54**, 77.

FIG. 1



Comparison of the lowering of blood sugar level by oxophenarsine-treated insulin with the lowering induced by regular insulin at the same unit dose. Test conducted immediately after preparation. Avg mg % blood sugar of 30 rabbits for each curve.

each test (control and unknown) the blood sugar curve shown in Fig. 1 was obtained.

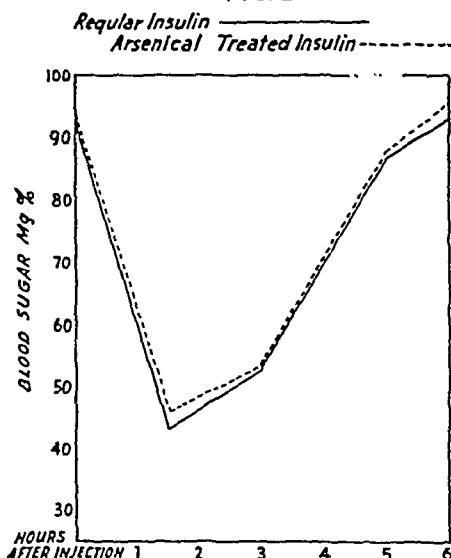
There was no difference in the 2 insulins at the 3rd hour, hence there was no potentiation, but the marked differences at the 5th and 6th hours were typical of an insulin with a delayed reaction.

To test the stability of the As-insulin complex the two specimens were again compared after standing in the icebox for 2 weeks. Two groups of 12 rabbits each were used. The blood sugar curve shown in Fig. 2 was obtained.

The arsenical combination was unstable and at 2 weeks standing in the cold the insulin had regained the hypoglycemic properties of regular insulin.

Discussion. Despite the instability of the oxophenarsine-insulin compound the evidence of interaction may be of interest to those studying the mechanism of action of insulin. It raises the question whether an arsenical interaction with S-S linkages has occurred, such as thioarsenite bridge formation, to delay the normal destruction of insulin by gluta-

FIG. 2



Repetition of the comparison after the arsenical-treated insulin had remained two weeks in the ice-box. Avg mg % blood sugar of 12 rabbits for each curve.

thione as postulated by DuVigneaud,⁴ or whether interaction might be with free amino groups of the hormone. Hallas-Moeller⁵ has reported that reaction of insulin with phenyl-isocyanate produced a delayed reaction insulin claimed to form by a reaction of the isocyanate compound with the histidine and lysine free amino groups. But Stern and White⁶ have claimed that acetylation of the free amino groups does not appreciably affect insulin activity, though acetylation of the tyrosine OH groups does. Whatever the mode of reaction may be, the compound formed is easily hydrolysed with regeneration of insulin. A further possibility is a brief, temporary partial inactivation of the insulin. At body temperature this effect may have been decreasing by the third hour, but be sufficient to produce

⁴ (a) du Vigneaud, V., Fitch, A., Pekarek, E., and Leekwood, W. W., *J. Biol. Chem.*, 1931, 94, 233; (b) du Vigneaud, V., *Cold Spring Harbor Symposia Quant. Biol.*, 1938, 6, 275.

⁵ Hallas Moeller, K., Dissertation, Copenhagen, 1945; via author's summary, *Arch. Pharm. exp. Chem.*, 1945, 52, 627.

⁶ Stern, K. G., and White, A., *J. Biol. Chem.*, 1936, 122, 371.

being converted to the arsonic acid, and that then another portion might react with insulin-SH to form thioarsenites. It was expected that such a reaction would cause loss of insulin potency. Under the proper conditions rather a prolongation of action but not a loss of potency was observed.

Methods. The insulin used was Lilly Isletin, 20 units/cc. The oxophenarsine hydrochloride was pure 3-amino-4-hydroxyphenyl arsenoxide hydrochloride hemialcoholate[†] (mol. wt. 254.4), and not the commercial Mapharsen, containing sucrose and sodium bicarbonate buffers.

The insulin bioassay method in preliminary tests was the 5-hour rabbit blood sugar method of Marks.² In later tests by the Eli Lilly Co. their standard method of 5-6-hour rabbit blood sugar test was used (tests at 1.5, 3, 5, and 6 hours).

Incubation of insulin and oxophenarsine: Into sterile rubber capped test tubes or bottles under sterile conditions a given volume of the insulin (*e. g.*, 10 cc of 20 units/cc) was delivered and an equal volume of sterile $n/320$ HCl in 0.85% saline was added. After replacement of the air with nitrogen gas the oxophenarsine salt in proper amount was dropped in, the container sterilely capped, shaken, and incubated for 18 hours at 37°C. The fluid was then used for the dilution (with the same sterile acid saline) to the 1 or $\frac{1}{2}$ unit per 0.5 cc dose injected into the rabbits. Control animals received the same insulin, which in the 1-1 dilution with acid saline was incubated in similar manner without addition of the arsenical (hereinafter called the regular insulin).

Calculation of the molar equivalent of oxophenarsine per molar sulfur content of the insulin: Assuming that 20 units of insulin equalled 2.32 mg of insulin and taking Svedberg's value for the mol. wt. of insulin at 37,000 and the sulfur content at 3.2%, it was calculated that the molecule of insulin contained 37 atoms of sulfur and that a mole of oxophenarsine would be needed for each. (It may be noted that Stern and White's³ claim of

36 atoms of sulfur, viz., 18 S-S links, would allow for a slight excess of oxophenarsine, while a higher mol. wt. with the same S percentage content would also allow an excess.) By calculation, with an equivalence of one mole of oxophenarsine per 37 atoms of sulfur, 1 mg of insulin would require 0.2534 mg of oxophenarsine salt, and 1 ml of 20 units/ml insulin would require 2.32×0.2534 or 0.5878 mg of the arsenical. Ten ml of 20 units/ml insulin would then need 5.89 mg for one mole, 11.78 mg for 2 moles. To allow a slight excess the above values were rounded to 6.00 mg and 12.00 mg respectively per 10 ml of 20 units/ml insulin.

Results. Preliminary work. An initial test by the Marks method, upon addition of 6.00 mg of oxophenarsine salt per 10 ml of 20 units/ml insulin and incubation, showed an apparent loss of potency from 100% for the regular insulin to 87% for the arsenically treated insulin.

In the expectation of a possible further loss of potency with a doubled quantity of the arsenical, that amount was tried and the incubate tested on 5 rabbits, which, on the first day received the regular insulin (1 unit) and 2 days later received the unknown "treated insulin" (1 unit). This statistically invalid test showed an apparent increase in potency such as might be found in working in the upper flat part of the dose response curve. Hence, another set of 5 rabbits was tested, using $\frac{1}{2}$ unit insulin doses, and again an apparent increase in potency was observed. At this point, however, the 6th hour blood sugars were taken and were found to average 26.1% below the normal at this time.

With the possibility that a delayed reaction insulin had been obtained, arrangements were made with Edward D. Campbell[‡] of the Eli Lilly Co. to prepare similar batches of control insulin and oxophenarsin-treated insulin and make a statistically valid test by their standard method.

Results of the Lilly Laboratories test: Based on results obtained from 30 rabbits on

[†] Kindly supplied by Dr. Oliver Kamm of the Parke Davis Company.

² Described in Burn, J. H., *Biological Standardizations*, London, 1938, p. 79ff.

³ Stern, K. G., and White, A., *J. Biol. Chem.*, 1937, **117**, 95; 1937, **119**, 215.

[‡] We desire to thank Dr. G. H. A. Clowes and Dr. Campbell for arranging these tests.

The hyaluronidase⁶ was obtained from bull testes according to the method of Hahn.⁸ The extract was dried while frozen and processed by the Madinaveitia method⁹ with ammonium sulfate fractionation. The final enzyme preparation contained 233 turbidity reducing units per milligram.¹⁰

Methods. Erythrocyte Sedimentation Rate. E.S.R. was measured throughout these experiments by a modified Westergren technic,¹¹ with no correction for hematocrit.

Estimation of Hyaluronic Acid. Many acid mucopolysaccharides will co-precipitate with protein at an acid pH.^{12,13} The resulting turbidity has been used by Kass and Seastone¹⁰ as a quantitative measure of hyaluronic acid; a modification of their procedure was employed. Serial dilutions of sodium hyaluronate were made in normal rabbit plasma, 1.0 ml samples of which were then acidified by adding 9.0 ml of pH 3.1 acetate buffer (0.5 M). Concentration of the sodium hyaluronate in the several samples could be correlated with percent transmission read in a photoelectric nephelometer¹⁴ with a 530 Å filter. In all cases, incubation of the serially diluted sodium hyaluronate with hyaluronidase (1.0 mg in 0.1 ml physiological salt solution) at 37° for one hour prior to acidification completely prevented the turbidity.

An attempt was made to use the carbazole reaction¹¹ to determine hyaluronic acid colorimetrically. By the direct method of Seibert and Atno,¹⁵ using galactose as the standard,

§ Kindly furnished for these experiments by courtesy of the Parke-Davis Company, Detroit, Mich.

⁶ Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

⁹ Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

¹⁰ Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **70**, 319.

¹¹ Westergren, A., *Acta Med. Scandinav.*, 1920, **54**, 247.

¹² Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

¹³ Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, 1937, **119**, 501.

¹⁴ Lumetron, Model 400A, Photovolt Corp., New York City.

¹⁵ Dische, Z., *Mikrochemie*, 1930, **8**, 4.

¹⁵ Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 1946, **163**, 511.

TABLE I.

Effect on Erythrocyte Sedimentation Rate of Adding Sodium Hyaluronate to Oxalated Rabbit Blood and Washed Erythrocyte Suspensions.

Sodium hyaluronate mg/ml	Erythrocyte sedimentation rate	
	Whole blood mm/hr	Washed erythrocytes* mm/hr
0.00	2	1
0.18	6	9
0.37	15	20
0.75	40	50
1.5	80	78
3.0	4†	0†

* Washed 3 times with physiological salt solution, then brought to volume in this diluent.

† Inhibition due to viscosity and clumping effects.

tests on serial dilutions of sodium hyaluronate in normal rabbit plasma showed that the procedure was not sensitive enough to measure accurately the small sodium hyaluronate concentrations needed to increase the E.S.R.

Preliminary Tests. To evaluate the reliability of the methods employed for the determination of hyaluronic acid in blood, experiments were carried out *in vitro* and *in vivo*. The increases obtained in E.S.R. on adding various dilutions of sodium hyaluronate to normal rabbit blood are shown in Table I. The apparent inhibition of the settling velocity in the highest concentration of the polysaccharide (3.0 mg/ml) was due to viscosity and clumping effects, since dilution with whole blood or physiological salt solution (P.S.S.) accelerated the E.S.R. Results similar to those in Table I were obtained with human blood. In every instance, following incubation with hyaluronidase, the settling velocity returned to normal. Hyaluronidase failed to affect the increased E.S.R. produced by gelatin or gum arabic.

In the *in vivo* studies, rabbits weighing 1 to 2 kg were injected intravenously with varying amounts of sodium hyaluronate. Increased E.S.R. and plasma turbidity could be demonstrated for varying periods after injection, as shown in Table II. In every case, the increased E.S.R. and the plasma turbidity were abolished by incubation with 1.0 mg of hyaluronidase at 37° for one hour.

Investigation of Blood from Clinical Cases. Specimens of oxalated blood were obtained

a delayed reaction.

Summary. On incubating insulin under sterile conditions in acid saline with 2 moles of oxophenarsine (3-amino-4-hydroxyphenyl-arsenoxide hydrochloride) for each atom of sulfur contained in the insulin, it was found (by blood sugar determinations) that the effect of the treatment was to prolong the

action on the blood sugar beyond that of untreated insulin. However, after 2 weeks standing this delayed action effect had disappeared and the material gave a blood sugar curve identical to that of the control insulin. Possible mechanisms of the interaction of oxophenarsine and insulin are briefly discussed.

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Failure to Relate Hyaluronic Acid to Elevated Erythrocyte Sedimentation Rate in Rheumatic Diseases.*

J. S. YOUNGNER AND C. H. ALTSHULER.† (Introduced by W. J. Nungester.)

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It has been demonstrated that many asymmetric macromolecular substances are capable of increasing the erythrocyte sedimentation rate (E.S.R.) when added to normal blood. The substances listed by Fahraeus¹ as having such an action were gelatin, agar, gum arabic, gastric mucin, fibrinogen, globulin, and sodium caseinate. In recent years, pneumococcus capsular polysaccharides,² desoxyribonucleic acid,^{3,4} and hyaluronic acid⁴ have been shown to exert a similar effect. Meyer reported^{4,5} that a testicular extract containing hyaluronidase, an enzyme complex which depolymerizes

hyaluronic acid, when added to the blood of rheumatic fever patients decreased the elevated E.S.R. to normal limits. This report seemed of significance since hyaluronic acid is believed to be a constituent of the amorphous ground substance of the connective tissue, the site primarily involved in rheumatic fever.⁶

The investigation reported here was undertaken to determine whether hyaluronic acid is present in the blood of patients with rheumatic diseases and whether this substance is responsible for the elevated E.S.R. commonly observed.

Materials. The sodium salt of hyaluronic acid was prepared by a modification of the method of Seastone.⁷ An aqueous extract of umbilical cord was deproteinized by stirring with a chloroform-amyl alcohol mixture in a Waring blender. The polysaccharide was finally precipitated by the addition of 2 volumes of ethyl alcohol saturated with sodium acetate in the cold. Sodium hyaluronate prepared in this manner produced a clear or opalescent, highly viscous solution at a concentration of 1.0%. To avoid discrepancies due to variations in the preparation, one batch was made and used throughout the experiment.

* Supported in part by the Faculty Research Fund of the Horace H. Rackham School of Graduate Studies, University of Michigan.

† Present address: J. S. Youngner, National Institute of Health, National Cancer Institute, Bethesda, Md.; C. H. Altshuler, Department of Pathology, University of Wisconsin, Madison, Wisc.

‡ The authors wish to thank Drs. W. D. Robinson and W. D. Block of the Rackham Arthritis Research Unit for furnishing blood from cases of rheumatoid arthritis and salicylate levels used in this report.

¹ Fahraeus, R., *Acta Med. Scandinav.*, 1921, 55, 1.

² Nungester, W. J., and Klein, L. F., *Proc. Soc. Exp. Biol. and Med.*, 1937, 36, 315.

³ Mori, S., *Kekkoku*, 1941, 19, 50.

⁴ Meyer, K., Hahnel, E., and Feiner, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, 58, 36.

⁵ Meyer, K., Chapter 18, *Currents in Biochemical Research*, ed. by Green, D. E., 1946.

⁶ Klinge, F., *Ergeb. d. allg. Path. u. Path. Anat.*, 1933, 27, 1.

⁷ Seastone, C. V., *J. Exp. Med.*, 1939, 70, 361.

The hyaluronidase[§] was obtained from bull testes according to the method of Hahn.⁸ The extract was dried while frozen and processed by the Madinaveitia method⁹ with ammonium sulfate fractionation. The final enzyme preparation contained 233 turbidity reducing units per milligram.¹⁰

Methods. Erythrocyte Sedimentation Rate. E.S.R. was measured throughout these experiments by a modified Westergren technic,¹¹ with no correction for hematocrit.

Estimation of Hyaluronic Acid. Many acid mucopolysaccharides will co-precipitate with protein at an acid pH.^{12,13} The resulting turbidity has been used by Kass and Seastone¹⁰ as a quantitative measure of hyaluronic acid; a modification of their procedure was employed. Serial dilutions of sodium hyaluronate were made in normal rabbit plasma, 1.0 ml samples of which were then acidified by adding 9.0 ml of pH 3.1 acetate buffer (0.5 M). Concentration of the sodium hyaluronate in the several samples could be correlated with percent transmission read in a photoelectric nephelometer¹⁴ with a 530 Å filter. In all cases, incubation of the serially diluted sodium hyaluronate with hyaluronidase (1.0 mg in 0.1 ml physiological salt solution) at 37° for one hour prior to acidification completely prevented the turbidity.

An attempt was made to use the carbazole reaction¹⁵ to determine hyaluronic acid colorimetrically. By the direct method of Seibert and Atno,¹⁵ using galactose as the standard,

§ Kindly furnished for these experiments by courtesy of the Parke-Davis Company, Detroit, Mich.

⁸ Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

⁹ Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

¹⁰ Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

¹¹ Westergren, A., *Acta Med. Scandinav.*, 1920, **51**, 247.

¹² Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

¹³ Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, 1937, **119**, 501.

¹⁴ Lumetron, Model 400A, Photovolt Corp., New York City.

¹⁵ Dische, Z., *Mikrochemie*, 1930, **8**, 4.

¹⁶ Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 1946, **163**, 511.

TABLE I.
Effect on Erythrocyte Sedimentation Rate of Adding Sodium Hyaluronate to Oxalated Rabbit Blood and Washed Erythrocyte Suspensions.

Sodium hyaluronate mg/ml	Erythrocyte sedimentation rate	
	Whole blood mm/hr	Washed erythrocytes* mm/hr
0.00	2	1
0.18	6	9
0.37	15	20
0.75	40	50
1.5	80	78
3.0	4†	0†

* Washed 3 times with physiological salt solution, then brought to volume in this diluent.

† Inhibition due to viscosity and clumping effects.

tests on serial dilutions of sodium hyaluronate in normal rabbit plasma showed that the procedure was not sensitive enough to measure accurately the small sodium hyaluronate concentrations needed to increase the E.S.R.

Preliminary Tests. To evaluate the reliability of the methods employed for the determination of hyaluronic acid in blood, experiments were carried out *in vitro* and *in vivo*. The increases obtained in E.S.R. on adding various dilutions of sodium hyaluronate to normal rabbit blood are shown in Table I. The apparent inhibition of the settling velocity in the highest concentration of the polysaccharide (3.0 mg/ml) was due to viscosity and clumping effects, since dilution with whole blood or physiological salt solution (P.S.S.) accelerated the E.S.R. Results similar to those in Table I were obtained with human blood. In every instance, following incubation with hyaluronidase, the settling velocity returned to normal. Hyaluronidase failed to affect the increased E.S.R. produced by gelatin or gum arabic.

In the *in vivo* studies, rabbits weighing 1 to 2 kg were injected intravenously with varying amounts of sodium hyaluronate. Increased E.S.R. and plasma turbidity could be demonstrated for varying periods after injection, as shown in Table II. In every case, the increased E.S.R. and the plasma turbidity were abolished by incubation with 1.0 mg of hyaluronidase at 37° for one hour.

Investigation of Blood from Clinical Cases. Specimens of oxalated blood were obtained

TABLE II.

Intravenous Injection of Sodium Hyaluronate in Rabbits. Inhibition of Increased Erythrocyte Sedimentation Rate and Plasma Turbidity by Hyaluronidase.

Rabbit	Sodium hyaluronate injected, mg/kg	Time after injection	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
			P.S.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S. % transmission	Hyaluronidase, % transmission
1	72.2	40 min.	72	1	54	100
		6 hr	15	5	69	100
		96 "	1	—	100	100
2	72.4	3 min	42	1	42	100
		1 hr	53	1	47	100
		6 "	15	1	62	100
3	38.7	30 min	12	1	61	100

* See text.

from 16 cases of rheumatic fever,¹⁷ 16 cases of rheumatoid arthritis, and 10 patients with various other diseases. The initial E.S.R. was first determined. To investigate the effect of hyaluronidase, 1.0 mg in 0.1 ml P.S.S. was added to 1.0 ml of the blood and as a control, 0.1 ml P.S.S. alone was added to an equal volume of blood. These specimens were incubated at 37°C for one hour and the E.S.R. again measured. As indicated in Table III, in most cases where there was a significant alteration of the E.S.R. by hyaluronidase, the P.S.S. alone produced a similar effect.

The turbidimetric method described earlier for estimating hyaluronic acid in plasma was applied to two 1.0 ml samples of plasma from the same blood. To one was added hyaluronidase; to the other P.S.S., as above. After incubation at 37°C for one hour, the plasmas were acidified with the acetate buffer. In no case did the two specimens show a significant difference in the turbidity formed. Rarely was there a measurable difference between the results obtained with the pathologic specimens and those from normal individuals.

It has been reported^{16,17} that many blood specimens contain antinvasin, a substance which inhibits hyaluronidase activity. Hyaluronidase inhibition by human or rabbit plasma

was not encountered under the experimental conditions in this study.

Many patients involved in this study had been on salicylate therapy. Since this therapeutic agent has been reported to have a hyaluronidase-inhibiting activity,^{18,19,20} experiments were carried out to determine whether it had been responsible for the failure of hyaluronidase to alter the E.S.R.

Varying quantities of sodium salicylate (10% in P.S.S.) and sodium hyaluronate were injected intravenously into rabbits, blood was drawn by cardiac puncture at intervals, and salicylate determined by the method of Brodie *et al.*²¹ Although salicylate affected the elevated E.S.R. produced by sodium hyaluronate, it did not alter hyaluronidase inhibition of E.S.R. and plasma turbidity (Table IV). This is in agreement with the results of Pike²² and would seem to rule out salicylate inhibition as a factor in the failure of hyaluronidase to alter significantly the E.S.R. of the clinical blood specimens tested. These findings do not rule out the possibility of interference with the oligosaccharase action of the hyaluronidase

¹⁸ Guerra, F., *Science*, 1946, **103**, 686.

¹⁹ Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

²⁰ Dorfman, A., Reimers, E. J., and Ott, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 357.

²¹ Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Therap.*, 1944, **80**, 114.

²² Pike, R. M., *Science*, 1947, **105**, 391.

¹⁷ Obtained through the courtesy of Dr. P. V. Wooley of Children's Hospital, Detroit, Mich.

¹⁶ Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

¹⁷ Haas, E., *J. Biol. Chem.*, 1946, **163**, 101.

TABLE III.

Effect of Hyaluronidase on Elevated Erythrocyte Sedimentation Rate of Blood from Patients with Various Diseases.

Disease condition	Patient	Erythrocyte sedimentation rate		
		Initial, mm/hr	After incubation with	
			Hyaluronidase,* mm/hr	P.S.S.,* mm/hr
Rheumatic fever	1	68	90	83
	2	113	122	115
	3	42	46	37
	4	95	71	90
	5	29	22	14
	6	18	8	7
	7	8	3	2
	8	19	8	8
	9	18	18	30
	10	74	67	55
	11	44	47	—
	12	45	24	16
	13	34	34	25
	14	26	8	10
	15	16	3	3
	16	17	9	5
Rheumatoid arthritis	17	52	34	34
	18	76	79	74
	19	106	122	112
	20	88	62	53
	21	40	40	36
	22	20	12	12
	23	20	15	2
	24	92	86	75
	25	38	42	37
	26	—	35	32
	27	38	33	32
	28	65	52	48
	29	85	80	63
	30	—	72	69
	31	105	107	105
	32	28	29	30
Acute disseminated lupus erythematosus	33	67	55	70
Upper respiratory infection	34	44	41	37
Pulm. tuberculosis	35	30	42	34
	36	62	88	79
Hodgkins disease	37	140	135	127
	38	95	110	95
Lymphosarcoma	39	52	27	27
Aplastic anemia	40	130	140	130
Pernicious anemia	41	41	29	34
	42	59	57	59

* See text.

complex.²³ Salicylates have been reported to decrease the E.S.R. of pathologic blood speci-

mens.²⁴ The explanation of this phenomenon is obscure and is under further investigation.

²³ Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1946, **22A**, No. 1; *Ibid.*, 1946, **22A**, No. 2.

²⁴ Homburger, F., *Am. J. Med. Sc.*, 1945, **210**, 168.

TABLE IV.

Failure of Sodium Salicylate to Alter Action of Hyaluronidase on Increased Erythrocyte Sedimentation Rate and Plasma Turbidity Following Intravenous Injection of Sodium Hyaluronate.

Rabbit	Sodium hyaluronate, mg/kg	Time after injection	Salicylate level, mg %	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
				P.S.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S., % transmission	Hyaluronidase, % transmission
1	91.7	30 min.	37.5	1	1	37	100
		5 hr	16.7	2	2	90	100
2	66.6	1 "	5.6	38	1	48	100
		2 "	4.6	10	1	66	100
3	62.5	1 "	52.8	15	2	52	100
		5 "	33.4	12	2	66	100
4	82.3	1 "	73.8	1	1	53	100
		5 "	43.0	12	1	63	100

* See text.

Discussion. The data presented in this report fail to substantiate the possibility that hyaluronic acid, at least in a polymerized form, is present in the blood of patients with rheumatic fever, rheumatoid arthritis, and several other diseases (Table III) as has been suggested by Meyer.^{4,5} The polysaccharide could not be demonstrated in the blood of normal individuals.²⁵ Depolymerized hyaluronic acid would not be demonstrated by the methods employed here, but previous work¹ and our own indicate that the depolymerized acid will not increase E.S.R. to a marked de-

gree. In a personal communication, Dr. Karl Meyer has stated that in studies with his preparations of hyaluronidase, spreading activity and E.S.R. inhibition did not run parallel. He was able to separate the factor that decreased E.S.R. from hyaluronidase activity by adsorption on lead sulfide.

Summary. 1. Hyaluronic acid is apparently not responsible for the increased erythrocyte sedimentation rate of blood from patients with rheumatic fever and rheumatoid arthritis. 2. Sodium salicylate failed to inhibit the effect of hyaluronidase on elevated E.S.R. produced in rabbits by the intravenous injection of sodium hyaluronate.

²⁵ Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

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Immunologic Reactions Following the Intradermal Inoculation of Influenza A and B Vaccine.*

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It has been shown¹ that the intradermal injection of heat-inactivated mumps virus into

human beings may exert a pronounced antigenic effect. Data are² available which sug-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc. The technical assistance of Sam Lavin and Alice H. Northrop is gratefully acknowledged.

¹ Enders, J. F., Kane, L. W., Maris, E. P., and Stokes, J., *J. Exp. Med.*, 1946, **84**, 341.

² Stokes, J., and Enders, J. F., 1947, unpublished data.

gest that this procedure may also induce active immunity. More recently, Van Gelder and associates³ compared the rise in anti-hemagglutinins following intradermal and subcutaneous inoculation of concentrated vaccine prepared from strains of A and B influenza viruses. Although the intradermal dose was only one-tenth as large as the subcutaneous dose, the smaller dose induced a greater mean antibody response. The incidence of generalized reactions was lower in the group which received intradermal inoculations. Localized reactions characterized by redness and swelling were observed in 90% of the subjects on the day following the intradermal injection. Previously, Beveridge and Burnet⁴ had noted immediate (10-20 minutes) and delayed (24 hours) dermal responses in certain individuals to intracutaneous injection of inactivated influenza virus. Although in adults they found no correlation between the antibody titer and the delayed reaction, in children the occurrence of the latter appeared to be associated with the presence of antibody.

In the early spring of 1947, an epidemic of influenza appeared imminent in Boston. Intradermal vaccination was then carried out in a group of adult hospital personnel. Information was obtained in respect to the antibody response following vaccination. The occurrence of local and generalized reactions was recorded as well as the incidence of upper respiratory infection following vaccination. These data are presented below.

Materials and Methods. The group consisted of persons working in the Children's Hospital, Boston. On March 19 and 20, 1947, 495 adults were interviewed and a history taken regarding allergic manifestations and previous influenza vaccinations; 24 individuals were eliminated because of a history of severe allergy and 4 because of a history of reaction to previous influenza vaccination. The remainder were given intracutaneously 0.1 cc of a 1:5 dilution in 0.85% salt solution of mixed A and B influenza vaccine (prepared

of Lederle Laboratories, Lot No. 2032-34A). Twenty minutes after vaccination, each person was examined and the extent of any erythema and induration at the site of inoculation recorded. Between 44 and 52 hours after vaccination, 449 individuals were re-interviewed, a history obtained of any systemic reaction, and the local reaction was measured. Before vaccination venous blood specimens were obtained from 110 individuals selected at random; 3 weeks after vaccination 88 of these persons were re-bled and questioned as to the occurrence in the interim of attacks of respiratory disease.

Between April 25 and May 5, 1947, questionnaires were filled out by 375 persons concerning the nature of any respiratory disease which occurred subsequent to vaccination. Data were obtained regarding the date of onset, duration, severity, and the presence or absence of the following symptoms: headache, fever, chills, sweating, cough, running nose, pain in eyes, muscle aches, sore throat, and general weakness. During the same period similar questionnaires were filled out by another group of 405 hospital workers who had not received influenza vaccine in March, 1947.

The paired specimens of serum were examined by a modification, developed at the Army Medical School,⁵ of the Hirst agglutination-inhibition test using the PR8 strain of influenza A virus and the Lee strain of influenza B virus. Instead of human type "O" cells 0.5% chicken cells were employed.

(1) *Local and Systemic Reactions.* The immediate and delayed cutaneous responses were similar to those described by Beveridge and Burnet.⁴ Twenty minutes after inoculation, in those who reacted, a moderate erythema of varying extent appeared around the site with occasionally a small central papule or wheal. The majority also showed after 48 hours a delayed reaction which consisted of a diffusely erythematous and slightly indurated area with a fairly well defined margin; at this time, however, in many persons the reaction had started to subside. The maximum diameters of the local reaction are classified in Table I.

Seventy-five or 16.7% of 449 individuals

⁵ Bull. U. S. Army Med. Dept., 1946, 6, 777.

³ Van Gelder, D. W., Greenspan, F. S., and Dufresne, N. E., *U. S. Naval Med. Bull.*, 1947, 47, 197.

⁴ Beveridge, W. I. B., and Burnet, F. M., *Med. J. Australia*, 1944, 1, 85.

TABLE I.

Maximum Diameter of the Immediate and Delayed Cutaneous Responses in 449 Adults Following Intracutaneous Vaccination.

Diameter of erythema, mm	Reaction after 20 min		Reaction after approx. 48 hr	
	No.	%	No.	%
0-4	33	7.3	26	5.8
5-9	43	9.6	40	8.9
10-14	68	15.2	30	6.7
15-19	58	12.9	31	6.9
20-24	64	14.2	35	7.8
25-29	79	17.6	51	11.4
30-34	63	14.1	76	16.9
35-39	22	4.9	43	9.6
40-44	11	2.5	60	13.2
45-49	4	0.9	18	4.0
50-54	2	0.4	22	4.9
55-59	0	0	6	1.3
60-64	1	0.2	3	0.7
65-69	0	0	3	0.7
70 or over	1	0.2	5	1.1
Total reactions > 10 mm	373	83.1	383	85.3

TABLE II.

Frequency of Symptoms Reported by 75 Individuals Who Complained of a Systemic Reaction Within 48 Hours After Intracutaneous Vaccination.

Symptoms	No. of times
Malaise	31
Headache	28
Fatigue	24
Nausea	14
Chills	9
Fever	6
Muscle pain	4
Sweating	1
Urticaria	1

reported that they had experienced a systemic reaction within 48 hours after vaccination. With one exception, the symptoms which are summarized in Table II were very mild. One person requested medical attention because of chills, malaise, and a temperature of 100.3°F.

(2) *Antibody Response Following Intracutaneous Vaccination.* Specimens of serum were obtained immediately before intracutaneous vaccination and again 3 weeks thereafter from 39 individuals who had not been previously vaccinated against influenza and who were free from respiratory disease during this 3-week period. The differences between the pre- and post-vaccination titers are recorded in Table III. From these data it is

apparent that the geometric mean of the anti-hemagglutinin titer rose $3.6 \times$ for Influenza A and $2.9 \times$ for Influenza B following vaccination. If those individuals whose initial titers were over 1:64 are excluded, it has been calculated that the geometric mean titer of 27 persons rose $5 \times$ for Influenza A (1:38 to 1:193) and that of 29 persons $3.2 \times$ for Influenza B (1:28 to 1:89).

Sixteen additional individuals who had reported having a respiratory infection during the period between bleedings showed a rise of the geometric mean titer against Influenza B from 1:38 to 1:134. This group is considered separately because of the presence of respiratory infection in the community, as noted below. However, since there was no evidence of infection with type B virus, there can be little doubt that the rise in titer occurred as a result of vaccination.

(3) *Incidence of Respiratory Disease in the Vaccinated and Unvaccinated Groups.* From the second to the fifth weeks following vaccination there was an increased incidence of respiratory disease in the community. The increase, in part at least, could be attributed to a virus resembling Influenza A as indicated by a significant rise in agglutinin-inhibition titer against the PR-8 strain in the sera of a few unvaccinated patients that were studied and by the isolation of atypical strains of Influenza A virus by other workers in Boston.⁶ But analysis of the questionnaires revealed very few cases in which a diagnosis of influenza could be made on the basis of the symptoms reported. Therefore the incidence of all acute upper respiratory infection was determined in the vaccinated and in the control groups. Of 316 individuals who received intracutaneous vaccine and who had not been previously vaccinated against influenza, 109 or 34% reported symptoms of an acute upper respiratory disease; in a comparable unvaccinated control group, 85 or 28% of 329 individuals had an acute respiratory disease.

(4) *Antibody Titer and Local and Systemic Responses to Intracutaneous Vaccination.* No correlation could be discerned between the antibody titer at the time of vaccination

⁶ Finland, M., and Morgan, H. R., 1947, personal communication.

TABLE III.
Changes in the Titer of Antihemagglutinins Against Influenza A and B Virus During a 3-Week Period Following Intracutaneous Vaccination.*

Antigen	Reciprocal of agglutinin inhibition titer	No. of individuals before vaccination	No. of individuals after vaccination	Fall	No. showing change in titer				
					No change	Rise in titer			
						2x	4x	8x	16x 32x
PR-8	<16	0	0	0	0	0	0	0	0
	16	7	0	0	0	0	0	3	1
	32	6	0	0	0	2	2	1	0
	64	14	2	0	0	6	6	2	0
	128	8	13	0	2	5	1	0	0
	256	2	15	0	0	2	0	0	0
	512	2	8	0	1	1	0	0	0
Lee	1024	0	1	0	0	0	0	0	0
	Total 39								
	Geom. mean titer 1:256		Geom. mean titer 1:256						
	<16	6	0	0	0	3	0	2	1
	16	8	3	0	0	0	5	3	0
	32	7	3	0	2	2	2	1	0
	64	8	8	0	2	4	1	1	0
Previously vaccinated individuals and those with respiratory infection during the serological period were excluded.	128	8	16	0	4	0	0	0	0
	256	2	7	1	0	1	0	0	0
	512	0	2	0	0	0	0	0	0
	Total 39								
	Geom. mean titer 1:35		Geom. mean titer 1:40						

* Previously vaccinated individuals and those with respiratory infection during the serological period were excluded.

and the size of either the immediate or delayed skin reaction. Thus, in 15 previously unvaccinated subjects in whom the agglutinin-inhibition titer against both PR-8 and Lee antigens was 1:32 or less, the average maximum diameter of the delayed skin reaction was 34 mm with extremes of 8 and 50 mm. In 16 similar persons with titers against both PR-8 and Lee of 1:64 or higher, the average maximum diameter was 36 mm with extremes of 10 mm and 60 mm.

Fifty-four of the 449 persons who were inoculated intradermally had previously received influenza vaccine; 39, or 72%, exhibited immediate skin reactions of 20 mm or greater. In contrast, comparable immediate reactions were observed in 205, or 52%, of those vaccinated for the first time. No relationship was noted between the size of the delayed skin reaction and previous vaccination.

No correlation was found between the extent of the delayed skin reaction and the antibody response following vaccination. The average size of the immediate and of the delayed skin reactions did not differ significantly in the group that reported a systemic reaction and in the group that had no systemic response.

Discussion. The results of the tests for changes in antihemagglutinin against Influenza B following intradermal vaccination with inactivated virus showed that when the initial titer of the antibody was low (*i.e.* 1:64 or less), the antigenic effect was appreciable. When the initial titer was moderate or high the response was slight or absent. Inactivated Influenza A vaccine introduced intracutaneously also appeared to exert a comparable, if not a greater, antigenic effect in persons with initial low titers. The fact, however, that an epidemic caused by an atypical Influenza A virus was current at the time this study was carried out prevents a categorical statement in respect to the effect of this antigen since the possible occurrence of inapparent infection cannot be eliminated. Failure of influenza vaccine to stimulate large increases in the antihemagglutinin titer when the initial titer is high has been noted by others after subcutaneous inoculation.⁷

The serologic data indicate that one-fiftieth of the dose of vaccine usually administered by the subcutaneous route when injected into the skin may give rise to antibody levels which are only slightly lower than those reported for the standard subcutaneous dose.^{8,9,10,11} In respect to the effectiveness of this route of inoculation our findings are in general in agreement with the observations made by Van Gelder and his associates³ on influenza vaccine and with those of Enders and his co-workers¹ on inactivated mumps virus. Quantitatively, though, Van Gelder and his associates obtained larger mean increases in titer for both A and B antigens. This difference may possibly be attributed to their employment of undiluted vaccine whereas in our experiments the vaccine was diluted 1:5. Furthermore, they noted that the antibody concentration continued to increase during the period from 2 to 4 weeks after vaccination. Our final determinations were made 3 weeks after inoculation.

In view of the small quantity of virus inoculated the high incidence of systemic reactions which were reported was unexpected. With one exception, however, these reactions caused no loss of working time. It is possible that a large number of very mild reactions were revealed by the method of personal interview which was employed.

Immediate and delayed local erythematous reactions of 10 mm or greater in diameter were encountered in over 80% of the subjects. The immunologic mechanisms underlying these reactions remain obscure. They may be either manifestations of hypersensitivity established by previous experience with the viruses or the effect of a specific toxic action of the vaccines. An unknown proportion of the immediate type might be occasioned by a

⁷ Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., *J. Exp. Med.*, 1942, **75**, 495.

⁸ Salk, J. E., Menke, W. J., and Francis, T., Jr., *Am. J. Hygiene*, 1945, **42**, 57.

⁹ Rickard, E. R., Thigpen, M., and Crowley, J. H., *Am. J. Hygiene*, 1945, **42**, 12.

¹⁰ Eaton, M. D., and Meiklejohn, G., *Am. J. Hygiene*, 1945, **42**, 28.

¹¹ Hirst, G. K., Plummer, N., and Friedewald, W. F., *Am. J. Hygiene*, 1945, **42**, 45.

non-specific irritative effect of the inoculum.

No evidence for an increased resistance attributable to the vaccination was obtained. The incidence of all types of upper respiratory disease during the 2-month period succeeding vaccination in both inoculated and uninoculated groups did not differ significantly. Several explanations may be offered for the ineffectiveness of the vaccine. The strains of Influenza A may have differed in antigenic composition from the prevailing epidemic strain. This possibility has been invoked to explain the relative ineffectiveness of vaccination in other parts of the country during the epidemic of last spring.^{12,13,14} The quantity of vaccine administered may have been too small to establish resistance. Vaccination may have been undertaken too late, since serologic evidence was obtained that Influenza A was present among the hospital personnel within ten days following the administration of the vaccine.

Although the prophylactic value of intradermal vaccination has not been demonstrated, additional experiments would seem desirable in view of the results of Van Gelder and his co-workers and those reported in this communication.

¹² Francis, T., Jr., Salk, J. E., and Quilligan, J. J., *Am. J. Public Health*, 1947, **37**, 1013.

¹³ Sigel, M. M., Shaffer, F. W., and Henle, W., *J. Bact.*, 1947, **51**, 277.

¹⁴ Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

Summary. 1. Intradermal inoculation into 39 adults of 0.02 ml of concentrated Influenza A and B vaccine gave rise to a mean antibody response (antihemagglutinin) against Influenza B virus which approached that obtained by others following the subcutaneous injection of 1.0 ml of undiluted vaccine. A greater mean increase in antibody against Influenza A was observed in the same group, although this increase could not be attributed conclusively to the effect of the vaccine.

2. The intradermal injection of 0.02 ml of concentrated vaccine into 449 adults was followed within 20 minutes by a local erythematous reaction exceeding 10 mm in diameter in 373 or 83.1%. In 383 or 85.3% of the same group delayed local erythematous reactions exceeding 10 mm in diameter were present after approximately 48 hours accompanied in many cases by slight induration.

3. No correlation could be established between the intensity of the immediate or delayed dermal reaction and the level of antihemagglutinin in the blood.

4. Mild systemic reactions were reported by 75 (16.7%) of 449 vaccinated persons.

5. Although cases of Influenza A infection were demonstrated in the community shortly after vaccination, the rate of all upper respiratory disease during the succeeding 2-month period in a group of 316 vaccinated persons was not significantly different from the rate among 329 unvaccinated persons working in the same institution.

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Use of the Spectrophotometer for Measuring Melanin Dispersion in the Frog.

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It is well established that melanophore changes in the frog are related to environmental conditions as well as endocrine factors within the animal. However, there apparently is no satisfactory data on how completely a dispersion or concentration of mela-

nin in the skin will interfere with the reflectance of light by the other pigments present.

A review of the literature regarding methods for measuring melanin dispersion or concentration in the skin indicates that most of the observations made were patterned after

and the size of either the immediate or delayed skin reaction. Thus, in 15 previously unvaccinated subjects in whom the agglutinin-inhibition titer against both PR-8 and Lee antigens was 1:32 or less, the average maximum diameter of the delayed skin reaction was 34 mm with extremes of 8 and 50 mm. In 16 similar persons with titers against both PR-8 and Lee of 1:64 or higher, the average maximum diameter was 36 mm with extremes of 10 mm and 60 mm.

Fifty-four of the 449 persons who were inoculated intradermally had previously received influenza vaccine; 39, or 72%, exhibited immediate skin reactions of 20 mm or greater. In contrast, comparable immediate reactions were observed in 205, or 52%, of those vaccinated for the first time. No relationship was noted between the size of the delayed skin reaction and previous vaccination.

No correlation was found between the extent of the delayed skin reaction and the antibody response following vaccination. The average size of the immediate and of the delayed skin reactions did not differ significantly in the group that reported a systemic reaction and in the group that had no systemic response.

Discussion. The results of the tests for changes in antihemagglutinin against Influenza B following intradermal vaccination with inactivated virus showed that when the initial titer of the antibody was low (*i.e.* 1:64 or less), the antigenic effect was appreciable. When the initial titer was moderate or high the response was slight or absent. Inactivated Influenza A vaccine introduced intracutaneously also appeared to exert a comparable, if not a greater, antigenic effect in persons with initial low titers. The fact, however, that an epidemic caused by an atypical Influenza A virus was current at the time this study was carried out prevents a categorical statement in respect to the effect of this antigen since the possible occurrence of inapparent infection cannot be eliminated. Failure of influenza vaccine to stimulate large increases in the antihemagglutinin titer when the initial titer is high has been noted by others after subcutaneous inoculation.⁷

The serologic data indicate that one-fiftieth of the dose of vaccine usually administered by the subcutaneous route when injected into the skin may give rise to antibody levels which are only slightly lower than those reported for the standard subcutaneous dose.^{8,9,10,11} In respect to the effectiveness of this route of inoculation our findings are in general in agreement with the observations made by Van Gelder and his associates³ on influenza vaccine and with those of Enders and his co-workers¹ on inactivated mumps virus. Quantitatively, though, Van Gelder and his associates obtained larger mean increases in titer for both A and B antigens. This difference may possibly be attributed to their employment of undiluted vaccine whereas in our experiments the vaccine was diluted 1:5. Furthermore, they noted that the antibody concentration continued to increase during the period from 2 to 4 weeks after vaccination. Our final determinations were made 3 weeks after inoculation.

In view of the small quantity of virus inoculated the high incidence of systemic reactions which were reported was unexpected. With one exception, however, these reactions caused no loss of working time. It is possible that a large number of very mild reactions were revealed by the method of personal interview which was employed.

Immediate and delayed local erythematous reactions of 10 mm or greater in diameter were encountered in over 80% of the subjects. The immunologic mechanisms underlying these reactions remain obscure. They may be either manifestations of hypersensitivity established by previous experience with the viruses or the effect of a specific toxic action of the vaccines. An unknown proportion of the immediate type might be occasioned by a

⁷ Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., *J. Exp. Med.*, 1942, **75**, 495.

⁸ Salk, J. E., Menke, W. J., and Francis, T., Jr., *Am. J. Hygiene*, 1945, **42**, 57.

⁹ Rickard, E. R., Thigpen, M., and Crowley, J. H., *Am. J. Hygiene*, 1945, **42**, 12.

¹⁰ Eaton, M. D., and Meiklejohn, G., *Am. J. Hygiene*, 1945, **42**, 28.

¹¹ Hirst, G. K., Plummer, N., and Friedewald, W. F., *Am. J. Hygiene*, 1945, **42**, 45.

non-specific irritative effect of the inoculum.

No evidence for an increased resistance attributable to the vaccination was obtained. The incidence of all types of upper respiratory disease during the 2-month period succeeding vaccination in both inoculated and uninoculated groups did not differ significantly. Several explanations may be offered for the ineffectiveness of the vaccine. The strains of Influenza A may have differed in antigenic composition from the prevailing epidemic strain. This possibility has been invoked to explain the relative ineffectiveness of vaccination in other parts of the country during the epidemic of last spring.^{12,13,14} The quantity of vaccine administered may have been too small to establish resistance. Vaccination may have been undertaken too late, since serologic evidence was obtained that Influenza A was present among the hospital personnel within ten days following the administration of the vaccine.

Although the prophylactic value of intradermal vaccination has not been demonstrated, additional experiments would seem desirable in view of the results of Van Gelder and his co-workers and those reported in this communication.

¹² Francis, T., Jr., Salk, J. E., and Quilligan, J. J., *Am. J. Public Health*, 1947, **37**, 1013.

¹³ Sigel, M. M., Shaffer, F. W., and Henle, W., *J. Bact.*, 1947, **51**, 277.

¹⁴ Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

Summary. 1. Intradermal inoculation into 39 adults of 0.02 ml of concentrated Influenza A and B vaccine gave rise to a mean antibody response (antihemagglutinin) against Influenza B virus which approached that obtained by others following the subcutaneous injection of 1.0 ml of undiluted vaccine. A greater mean increase in antibody against Influenza A was observed in the same group, although this increase could not be attributed conclusively to the effect of the vaccine.

2. The intradermal injection of 0.02 ml of concentrated vaccine into 449 adults was followed within 20 minutes by a local erythematous reaction exceeding 10 mm in diameter in 373 or 83.1%. In 383 or 85.3% of the same group delayed local erythematous reactions exceeding 10 mm in diameter were present after approximately 48 hours accompanied in many cases by slight induration.

3. No correlation could be established between the intensity of the immediate or delayed dermal reaction and the level of anti-hemagglutinin in the blood.

4. Mild systemic reactions were reported by 75 (16.7%) of 449 vaccinated persons.

5. Although cases of Influenza A infection were demonstrated in the community shortly after vaccination, the rate of all upper respiratory disease during the succeeding 2-month period in a group of 316 vaccinated persons was not significantly different from the rate among 329 unvaccinated persons working in the same institution.

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Use of the Spectrophotometer for Measuring Melanin Dispersion in the Frog.

G. G. DEANIN AND F. R. STEGGERDA.

From the Department of Physiology, University of Illinois, Urbana, Ill.

It is well established that melanophore changes in the frog are related to environmental conditions as well as endocrine factors within the animal. However, there apparently is no satisfactory data on how completely a dispersion or concentration of mel-

nin in the skin will interfere with the reflectance of light by the other pigments present.

A review of the literature regarding methods for measuring melanin dispersion or concentration in the skin indicates that most of the observations made were patterned after

the general descriptive technic reviewed by Parker¹ in which the individual melanophores are given a numerical value corresponding to the various stages of expansion they may happen to be in at the time of examination, and which neglects to consider how effectively the contracted or expanded state of these melanophores will change the appearance of the frog or rest of the pigment normally present in the skin. It should also be pointed out that, even though the results obtained by this method for recording melanin dispersion or concentration in individual melanophores may be accurate and consistent, there always remains an unavoidable subjective factor when one attempts to catalogue melanophores in different stages of expansion in a microscopic field. It would therefore be highly desirable to have an objective method which would give quantitative information concerning the actual amount of masking the dilated melanophores will have over the rest of the skin pigments in the intact animal.

Although Hill and Solandt² and Smith,³ by attaching a photo-electric cell to a microscope, have recorded the variation in transmitted light through the skin and scales of fish under dark and light adapted conditions, they made no attempt to study the effects of melanin dispersion in the intact skin of the frog on the light reflected at each wave length of the visible spectrum. In a search for a method that would answer the requirement above mentioned, it was found that the Hardy⁴ photo-electric recording spectrophotometer would successfully give information on the kinds of pigments giving rise to the color of the skin as well as give an objective measurement of the amount of reflectance each pigment may give at its respective wave length when the melanophores are in an expanded or contracted state. The results obtained from experimentation with this technic showed

significant differences in skin reflectance in frogs that had previously been either light or dark adapted, and we herewith present the experimental technic used and the results obtained.

In preparing frogs for this type of recording 2 different sets of experimental conditions were used: one group of 8 frogs was exposed to light, and an equal number to dark for the same period of time. To accomplish light adaptation, the frogs were kept for a minimum of 72 hours in a white container which was illuminated at all times by two 40-watt bulbs; for dark adaptation the frogs were kept in a black container for 72 hours, also illuminated by two 40-watt bulbs. To record the skin reflectance the frogs, unanesthetized, were made immobile by fastening them to a frog board. They were then placed so that the back of the frog covered the round aperture (one inch in diameter) of the recording spectrophotometer. The light rays of various wave lengths of the visible spectrum were individually focused on the skin of the frog, and the amount of light reflected in each of the wave lengths was measured and recorded on ruled paper on a percentage reflectance basis. The spectrophotometer was moved by hand in preference to having the complete rotation of the drum made by the motor. The total time necessary for making a complete record was one minute, whereas, with the motor, a total time of 3 minutes was necessary. To insure accuracy the frogs were always placed in the same position before the light aperture of the instrument and the time of exposure was kept constant to avoid any inconsistencies so far as light from the spectrophotometer and melanophore responses were concerned.

The reflectance from the skin surface of the light adapted frogs is definitely greater in all wave lengths measured than the reflectance from the skin of the dark adapted frogs. This can be seen in the graph, which shows the average % reflectance at various wave lengths for light (upper curve) and dark (lower curve) adapted frogs. The results also indicate that melanophore expansion is apparently able to mask the reflecting power of the other pigments in the frog skin. It is

¹ Parker, G. H., *Biol. Bull.*, 1943, **84**, 273.

² Hill, A. V., and Solandt, D. Y., *J. Physiol.*, 1934, **83**, 13.

³ Smith, D. C., *J. Cell. and Comp. Physiol.*, 1936, **8**, 83.

⁴ Hardy, A. C., *J. Optic. Soc. America*, 1935, **25**, 305.

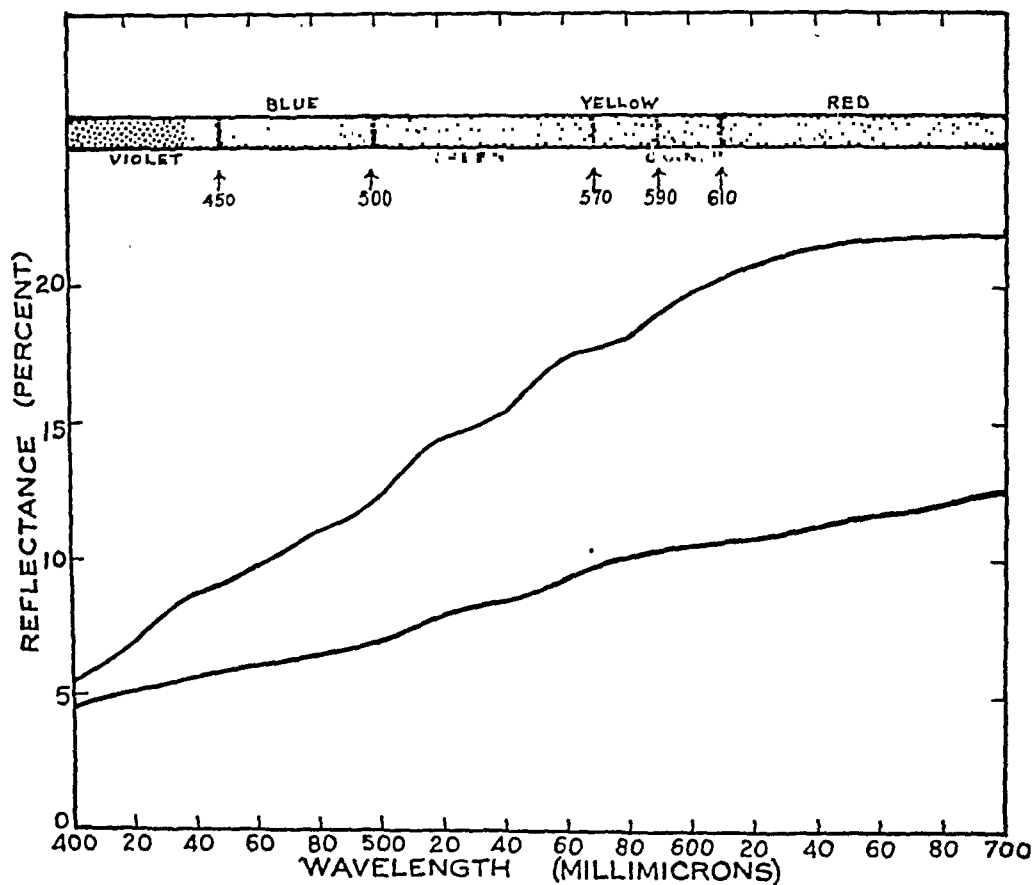


FIG. 1.

interesting to note that the general shape of the curve and degree of reflectance from the skin of the frog under these conditions compare quite closely with the degree of melanin distribution and concentration obtained by Edwards and Duntley⁵ from the dorsal surface of the hand of a Negro when examined with the same technic as used in these experiments. Furthermore, the reflectance from the skin surface of the frog is greater for wave lengths at the red end of the spectrum than at the violet end. Statistical analysis of the data justifies these conclusions. Although it is obvious from the data that there is a definite difference in reflectance between light and dark adapted frogs, it is rather surprising to find that the light adapted

frogs reflect an average of only 21.8% in the red end of the spectrum where the actual reflectance is at its maximum, while the dark adapted frogs, black as they appear to the eye in comparison, reflect an average of 12.3% at the same wave length. Surely these quantitative measurements show the inadequacy of our subjective observations.

In a few additional experiments, it was interesting to note that frogs made permanently pale as a result of complete hypophysectomy⁶ showed melanophores that did not constrict so much or allow for so great a reflectance as those in frogs that were light adapted. If the frogs were made permanently dark because of melanophore expansion following injury of the pars tuberalis region of

⁵ Edwards, E. A., and Duntley, S. Q., *Am. J. Anat.*, 1939, 65, 1.

⁶ Hogben, L. T., *Quart. J. Exp. Physiol.*, 1923, 13, 177.

the pituitary gland,⁷ the dilated state of the melanophores was greater and showed definitely less reflectance than frogs that were dark adapted by environmental stimulation only. Further studies of the same kind in which various dosages of related hormones are given may lead to helpful information concerning the mechanism of chromatophore action in animals.

Summary. (1) The spectrophotometer has been used as an instrument for recording the effects of melanophore expansion on the re-

fecting power of the skin pigments of the frog. (2) The reflectance in the light adapted frogs may be as much as 10% above that of the dark adapted frog at the same wave length. (3) Melanophore expansion in dark adapted frogs masks the reflecting power of the skin pigment at the red end of the spectrum more than at the violet. (4) The average skin reflectance in the light adapted frogs is not more than 21.8%. (5) A difference between the response of melanophores to environmental conditions and operations of the hypophysis is suggested.

⁷ Steggerda, F. R., and Soderwall, A. L., *J. Cell. and Comp. Physiol.*, 1939, **13**, 31.

16218

Interrelationship of Vitamin D and the Sex Hormones in Calcium and Phosphorus Metabolism of Rats.

G. F. NORMAN AND A. MITTLER. (Introduced by W. J. Kerr.)

From the Research Department, California Packing Corporation, San Francisco.

Introduction. The present therapeutic trend for treating chronic arthritis in humans involves the use of high dosages of vitamin D, and has many successes to its credit.^{1,2} The favorable treatment of Raynaud's disease in the same manner, in conjunction with sex hormones, has been reported.³ The high incidence of arthritis in women at a time near to or following the menopause, and the more gradual appearance of this disease in men, is well known. This leads to the suggestion that the role of the sex hormones in these relationships involving the therapeutic use of vitamin D should be more fully understood.

Hypertrophy of the parathyroids has been described in a number of suggestively analo-

gous conditions, viz.: rickets,⁴ scleroderma,^{5,6,7} Raynaud's disease,^{8,9} and chronic arthritis.¹⁰ Experimentally, by perfusion experiments,¹¹ a low calcium level in the perfusate leads to similar hypertrophy which is overcome by the addition of adequate amounts of calcium. Hyperphosphatemia also induces a corresponding enlargement as one would expect. Vitamin D reduces the compensatory hypertrophy induced by low calcium feedings.¹²

⁶ Leriche, R., and Jung, A., *La Presse Med.*, 1938, **46**, 809.

⁷ Leriche, R., *Gazette du Hospitalier*, 1936, **109**, 209; *Ab. J. A. M. A.*, 1936, **106**, 1214.

⁸ Bernheim, Alice R., *J. A. M. A.*, 1933, **100**, 1001.

⁹ Bernheim, Alice, and Garlock, J. H., *Ann. Surgery*, 1935, **101**, 1012.

¹⁰ Wootton, W. T., *J. Missouri M. A.*, 1936, **33**, 129; *Ab. J. A. M. A.*, 1936, **106**, 1854.

¹¹ Path, Harvey M., Wallerstein, Elizabeth, and Luckhardt, Arno B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 580.

¹² Carnes, William H., Pappenheimer, Alvin M., and Stoerk, Herbert C., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 314.

¹ Snyder, R. Garfield, Squires, Willard, Forster, John, Traefor, Cornelius, and Wagner, Lewis Clark, *Indust. Med.*, 1942, **11**, 295.

² Wagner, Lewis, *Industr. Med.*, 1942, **11**, 313.

³ Norman, G. F., *West. J. Surg., Ob. and Gyn.*, 1938, **46**, 553.

⁴ Nonidez, J. F., and Goodale, H. D., *Am. J. Anat.*, 1927, **38**, 319.

⁵ Cornbleet, T., and Struck, N. C., *Arch. Dermat. and Syph.*, 1937, **35**, 188.

It also further diminishes the size of these glands already reduced by hypophosphatemia while simultaneously raising both calcium and phosphorus levels in the blood serum.

This apparently occurs independently of the pituitary,^{13,14} and the control, if any, of this organ on the parathyroids must occur through the gonads via the gonadotropic hormones.

The effects of overdosage of the parathyroid hormone or the results of tumors of these glands on the organism are well known and need only passing mention here. The demineralization of bone shafts with deformities, the calcification of soft tissues, notably kidneys, lungs, stomach, and arteries, have been described in detail elsewhere.

The present tests were carried out in 1944 and 1945 to investigate the relationship above mentioned, and are now being reported because of the increasing importance of the use of vitamin D in the treatment of arthritis and other conditions. The results obtained were used to support the clinical work undertaken by the senior author.¹⁵

The present study was undertaken to determine the effect of the addition of estrogens and androgens to rachitic animals administered high doses of vitamin D.

(a) The effect of the addition of the estrogen and androgen were studied in homologous and heterologous fashion.

(b) Criteria of bone-ash were employed in fixing the effect of the addition of vitamin D to a rachitic animal, and also to measure the increment, if any, due to the dosing of the estrogen or androgen, as the case may be.

Experimental. The diet used was Rachitic Diet No. 2, as advocated in the United States Pharmacopoeia Twelfth Revision, supplemented with .1 g each of riboflavin and thiamine hydrochloride per 100 lb of diet. The vitamin D administered orally to the rats was the U. S. P. Reference Oil No. 2. The hormone supplements used were testosterone propionate in sesame oil (Ciba perandren), and estradiol di-propionate in sesame oil

(Ciba di-ovocycin). These supplements were injected subcutaneously. Where no hormone supplement was given the rats were injected with Wesson Oil.

All of the animals used in these experiments were of the Long-Evans strain bred from the colony maintained in this laboratory. The young animals had been raised in accordance with U. S. P. standards and were from groups which were available for U. S. P. vitamin D assays. The number of animals assigned to each group varied from 5 to 7 and the groups were adjusted to obtain approximately equal starting weights. The variability within groups due to litter was decreased by the use of isogenic segregation. Litters were chosen which had 3 to 5 animals of the same sex in order that one animal of each litter would appear in each group of a given study; for example: a triad of male rats from the same litter would be found in the control group receiving no vitamin D and no hormone, in the test group receiving only vitamin D, and in the test group receiving vitamin D and the hormone supplement. The rats were kept in individual wire-screen cages and given feed and water *ad libitum*.

In the course of these experiments bi-weekly weights of the animals were taken. At the end of the test period individual blood samples were taken as follows: An incision was made across the belly of the animal, cutting directly into the abdomen, and exposing the vena cava. The vena cava was then severed and the blood sample collected in a centrifuge tube. The chemical analysis of the blood serum was accomplished on a micro scale. Calcium was determined by the permanganate titration method of Tisdall.¹⁶ The inorganic phosphorus was determined by precipitation of the proteins with trichloroacetic acid, and a colorimetric determination was made after the reduction of the phosphomolybdic complex with stannous chloride, as advocated by Kuttner and Cohen.¹⁷

For bone-ash analysis the left tibiae were removed and freed of adhering tissue by buffing with cheesecloth. The bones were

¹³ Albright, Fuller, J. A. M. A., 1939, 112, 2592.

¹⁴ Carnes, Wm. H., Osebold, John, and Stoerk, Herbert C., *Am. J. Physiol.*, 1943, 139, 188.

¹⁵ Norman, G. F., *Geriatrics*, 1947, 2, 24.

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¹⁶ Tisdall, F. F., *J. Biol. Chem.*, 1923, **56**, 439.

¹⁷ Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517.

TABLE I.
Experiment I.
Effect of the Injection of Hormones in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

Depleted Rats Fed Various Doses of Vitamin D.							
Group	Vitamin D		Hormone dosage		Net gain		Tibia bone-ash %
	Daily dose, mg	Total fed, mg	Medication	Total dose, mg	Mean, g	Std. Dev.	
Males (Paired Litter Mates).							
1	0	0	Wesson Oil	0	139	± 3.87	31.12
2	6.0	210.0	" "	0	180	± 2.50	48.47
3	6.0	210.0	Perandren	17.5	148	± 4.03	46.22
7	.9	31.5	Wesson Oil	0	170	± 5.62	35.19
8	.9	31.5	Di-ovocylin	17.5	104	± 3.60	42.43
Females (Paired Litter Mates).							
4	0	0	Wesson Oil	0	179	±3.03	36.36
5	6.0	210.0	" "	0	203	± 5.03	50.69
6	6.0	210.0	Di-ovocylin	3.5	88	± 3.35	53.53
9	.9	31.5	Wesson Oil	0	235	± 5.74	44.66
10	.9	31.5	Perandren	17.5	212	± 4.51	38.16

extracted in anhydrous isopropanol for 2 hours and then ashed by group.

On the next to the last day of the test the animals were anesthetized and individual X-rays taken of each rat. Representative X-rays are presented to show the skeletal development which resulted from the studies undertaken.

In the preliminary studies young male rats were depleted for 18-21 days on the rachitic diet and then placed on assay for 10 days under conditions prescribed in the United States Pharmacopoeia. The daily dose of vitamin D was fed orally, and hormone supplements administered subcutaneously at the start of the assay and on the fifth day of assay. Results obtained indicated that although a large difference in percent bone-ash was obtained between the control group fed no vitamin D (29.50) and the highest level of vitamin D fed 10.67 mg per day (34.61), the 10-day test period would not allow for significant changes due to the injection of the hormone. It was felt that a longer assay period would allow for more treatments and, hence, a more critical study of the role of the hormone supplements. Therefore, in the assays herein reported the "prophylactic bone-ash method" as advocated by Coward¹⁸ was used. When the rats were weaned they were placed directly on the

rachitic diet and the assay started, continuing for 5 weeks. The dose of vitamin D was one-fifth of the curative test and given twice weekly instead of daily. The hormone supplement was injected every 5 days (Sundays excluded).

Results and Discussion. As advocated by Coward,¹⁸ the level of vitamin D to be fed in the "prophylactic bone-ash method" was one-fifth of the dose used in the curative U. S. P. assay required to give a 2-plus healing. The level of vitamin D used on assay designated as the high dose was 5 times the level required to give a 2-plus healing.

Sixty rats, ranging in age from 21-24 days, and in weight from 40-63 g, and consisting of 30 males and 30 females, were allotted into 10 groups, as shown in Table I. The 5 male groups were made up of litter-mate-brothers, and similarly the 5 female groups of litter-mate-sisters. One of the 5 groups served as the control for the 2 test groups involving the treatment with the homologous hormone, and also as the control for the other 2 groups devoted to the treatment with the heterologous hormone. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table I.

In order to determine statistically whether or not the mean difference observed in Table I between Groups 5 and 6 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings

¹⁸ Coward, K. H., *Biological Standardisation of the Vitamins*, Baillière, Tindall and Cox, London, 1938, 121.

TABLE II.
Experiment I.
Statistical Analysis of Data.

Group 5: Females; vitamin D—high dose.		
Group 6: Females; vitamin D—high dose plus Estrogen.		
	Tibia Bone-ash %	
Group 6		Group 5
56.72		43.73
51.83		49.09
53.85		46.94
53.79		49.09
51.21		51.94
54.04		54.43
Mean	53.57	49.20
Mean diff.		4.37
Stand. dev. of diffs.		5.14
Stand. error of mean diffs.		2.099
Critical ratio (t)		2.08

With 5 degrees of freedom the value for *t* when *P* equals .10 is 2.015, and the value found exceeds this limit.

except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 6 pairs of sisters the results obtained were treated according to "Student's" method of analysis for correlated data as shown in Table II.¹⁹

From Table I it will be seen that females tolerate depletion better than males, as indicated by the higher percent bone-ash for the control group (Group 4 vs. Group 1). This could possibly be explained by the presence of ovarian tissue.

It is obvious that the addition of a large amount of vitamin D led to an increase in body weight in both sexes, while the added medication counteracted the increase due to vitamin D.

In males the employment of estrogen significantly increased the percentage of bone-ash (Group 7 vs Group 8), while this was diminished with androgen as the medication (Group 2 vs. Group 3).

The use of the high dosage of vitamin D in the females definitely enhanced the percent bone-ash, and this was further increased with the use of the estrogen (Group 5 vs. Group 6). On the low dose of vitamin D the females were particularly sensitive to the androgen, definitely decreasing the percent bone-ash (Group 9 vs. Group 10). In this instance 2

factors apparently operate, namely, the low protection of the vitamin D dosage and the parathyroid stimulating effect of the androgen.

As shown in the statistical analysis in Table II, a significant increase in the percent bone-ash was observed when female rats given a high dose of vitamin D were concurrently treated with estrogen (Group 5 vs. Group 6).

In the first experiment with female rats the use of the estrogen definitely enhanced the percent bone-ash when given in connection with the high dose of vitamin D, and similarly showed an increase for males when the low dose of vitamin D was administered. On the other hand, the use of the androgen decreased the percent bone-ash when given in connection with the low dose of vitamin D to females, and also showed the same effect when given with the high dose of vitamin D to males.

These findings are in accord with the observations of Gardner,^{20,21} who investigated the effect of estrogens and androgens on the breaking strength of femurs of mice. However, the use of rachitic animals and the employment of sex hormones in conjunction with vitamin D have not been considered. In addition, the increase in bone-ash attributed to the use of the estrogen confirms the work of Day and Follis.²² It was proposed, therefore, to continue the study of the use of the androgen in connection with the high dose of vitamin D administered to both males and females.

Thirty-seven rats, ranging in age from 23-26 days, and in weight from 47-74 g, and consisting of 22 males and 15 females, were allotted into 6 groups, as shown in Table III. The 3 male groups were made up of litter-mate-brothers, and similarly the 3 female groups of litter-mate sisters. One of the 3 groups served as the control for the 2 test groups involving the treatment with per-andren.

The 3 male groups consisted of 8 animals

²⁰ Gardner, W. U., *Endocrinology*, 1943, **32**, 149.

²¹ Gardner, W. U., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 230.

²² Day, Harry G., and Follis, Richard H., Jr., *Endocrinology*, 1941, **28**, 83.

¹⁹ "Student," *Metron*, 1925, **5**, No. 3, 114.

TABLE III.
Experiment II.
Effect of the Injection of Androgen in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

					Analysis of serum				
Group	Vitamin D		Androgen dosage		Net gain		Tibia bone-ash, %	Calcium, Mean, mg %	Inorganic phosphorus, Mean, mg %
	Daily dose, mg	Total fed, mg	Medication	Total dose, mg	Mean, g	Std. Dev.			
Males (Paired Litter Mates).									
1	0	0	Wesson Oil	0	37.00	± 4.18	34.44	9.98	3.7
2	6	210	" "	0	37.43	± 4.70	49.03	10.57	3.6
3	6	210	Perandren	17.5	34.86	± 3.92	47.29	9.63	3.8
Females (Paired Litter Mates).									
4	0	0	Wesson Oil	0	33.20	± 1.48	35.57	9.20	3.8
5	6	210	" "	0	29.00	± 3.85	50.60	10.80	3.1
6	6	210	Perandren	17.5	28.20	± 3.25	49.09	9.54	3.9*

* Mean of 3 samples.

in the control and seven in each of the test groups, while the 3 female groups were made up of 5 animals each. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table III.

In the first experiment blood samples were taken, but were only used to establish the technic for handling the samples taken in this experiment. Therefore, in addition, Table III contains a summary of the analysis of the serum with respect to the calcium and inorganic phosphorus.

From Table III it will be observed that with both sexes there was a gain in weight in the animals employing vitamin D which was equal to the controls, as well as a definite increase in the percent bone-ash of the tibia. The use of androgen again diminished the percentage of bone-ash, and also reduced the net gain in weight when employed in conjunction with the vitamin D.

In the blood serum there were apparently no significant changes in the inorganic phosphorus level; however the calcium levels were depressed with the use of the androgen as the medication.

In order to determine statistically whether or not the mean difference observed in Table III between Groups 2 and 3 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 7 pairs

TABLE IV.
Experiment II.
Statistical Analysis of Data.

Group 2: Males; vitamin D—high dose.
Group 3: Males; vitamin D—high dose plus Androgen.

Tibia Bone-ash %		Group 3
Group 2		
50.35		49.29
45.78		46.39
48.39		46.12
50.84		51.26
46.50		46.63
50.78		47.83
47.69		44.69
Mean	48.62	47.46
Mean diff.		1.16
Stand. dev. of diff.		1.588
Stand. error of mean diff.		.600
Critical ratio (t)		1.933

With 6 degrees of freedom the value for *t* when *P* equals .10 is 1.943, and the value found approximates this limit.

of brothers the results obtained were treated according to "Student's" method for analysis for correlated data as shown in Table IV.¹⁹

The individual X-rays of the rats employed in the 2 tests were critically examined and the changes in bone structure noted. With respect to both sexes, the female sex hormone increased the density of the bone-shaft and indicated an advanced epiphyseal closure. The male sex hormone showed an opposite effect on both sexes as evidenced by a diminution of calcification of the bone-shaft and a slowed epiphyseal closure. These changes are best illustrated in representative photographs

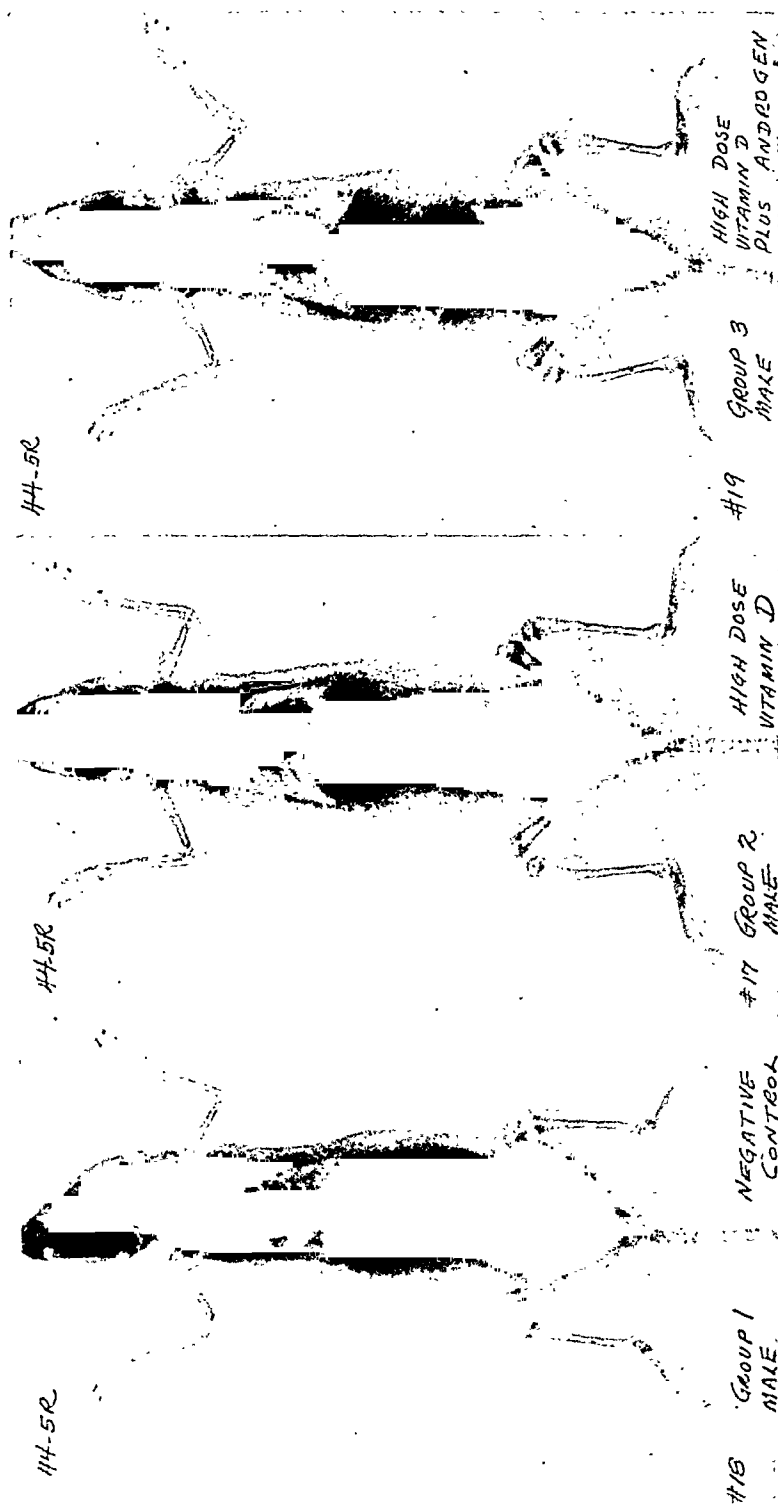


FIG. 1.

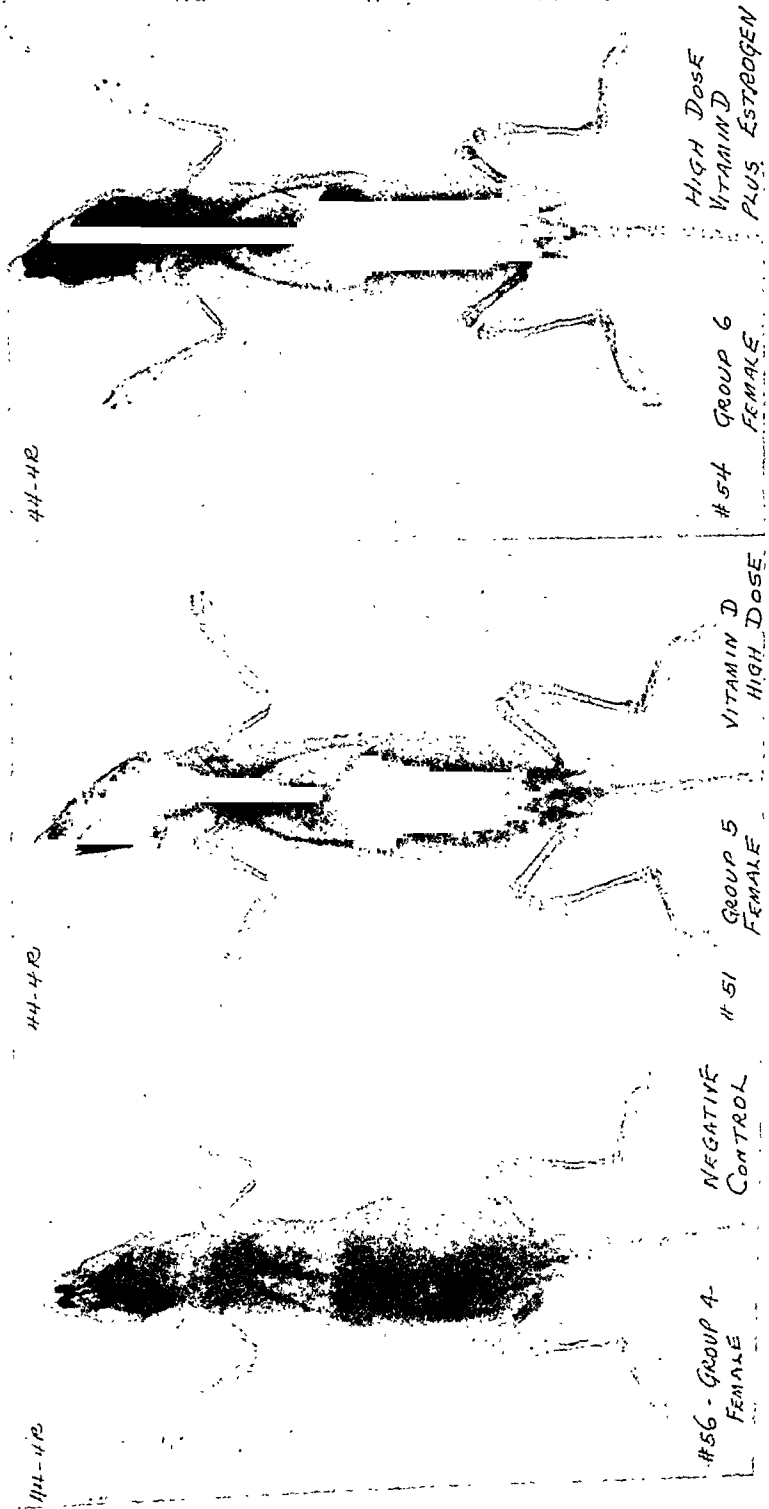


FIG. 2.

of sets of litter mates taken from the assay procedures.

Conclusion. On the basis of these experiments carried out with rats, it is our conclusion, in agreement with other observers, that for animals of both sexes the female sex hormone (a) stunts the growth of young animals, (b) increases the density of the bone-shaft, (c) accelerates epiphyseal closure, (d) increases the percentage of bone-ash.

These postulates confirm the depressing effect of this hormone on the parathyroid glands; further confirmed by the greater response in this effect when female animals were used (Experiment I).

The male hormone, on the other hand, in both sexes (a) stunts the growth of the animal, (b) diminishes the density of the

bone-shaft, (c) diminishes the percentage of bone-ash, (d) delays epiphyseal closure, (e) depresses slightly the blood calcium, but does not increase the serum inorganic phosphorus.

To summarize: The male sex hormone is rachitogenic; the female sex hormone is anti-rachitic. The use of the female hormone is suggested either alone or in conjunction with vitamin D in the treatment of either sex where there is overaction of the parathyroids.

The authors wish to thank the California Packing Corporation and Mr. J. E. McConkie, Director of Research of that organization, for their splendid cooperation in these vitamin studies, and to the Ciba Pharmaceutical Company for the liberal contribution of their endocrine products used in these studies.

16219 P

Activation of Plasma Thromboplastinogen and Evidence of an Inhibitor.*

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Evidence has been presented earlier indicating that the first reaction in the coagulation of the blood is the conversion of the precursor of thromboplastin, thromboplastinogen, to the active form.¹ This is brought about by a factor in the platelets. Hemophilic platelets appear equally as active as those of normal blood. The reaction can readily be studied by the prothrombin consumption test, which consists in determining the prothrombin before and after coagulation. Typical results are presented in Table I.

Recently Milstone² has also obtained data showing that the thromboplastin in plasma occurs in an inactive form which he names

prothrombokinase. He observed that it is activated in the presence of calcium, but he does not mention or apparently consider the platelets as a possible activation factor. From recent studies¹ it appears more likely that thromboplastin is not an enzyme but that its activation is enzymatic.

Recently it was found that the blood of a patient who developed a hemophilia-like disease (presumably as a sequela to pemphigus) contains an agent which inhibits the activation of thromboplastinogen. Significant results obtained in the study of the patient's blood are given in Table II. In addition it was found that the prothrombin time response of the patient's plasma to serial dilutions of thromboplastin is identical with that of normal plasma.

It seems clear that the impaired coagulation is not due to an anti-thromboplastin. The cause must be due either to a lack of thromboplastinogen or to failure of the latter

* This work was supported by a grant from the U. S. Public Health Service.

† Department of Internal Medicine, University of Rome; at present Senior Research Fellow, National Institute of Health.

¹ Quick, A. J., *Am. J. Med. Sci.*, 1947, **214**, 272.

² Milstone, J. H., *Science*, 1947, **106**, 546.

TABLE I.
Prothrombin Consumption in the Coagulation of Normal Blood, of Normal Deplateletized Plasma and of Normal Deplateletized Plasma Mixed with Hemophilic Platelets.

	One hour after coagulation	
	Prothrombin time of serum† sec	Prothrombin activity remaining %
Normal blood	37	10
Deplateletized* normal plasma	10½	100
Deplateletized normal plasma mixed with hemophilic platelets†	37	10

* The needle and syringe used for collecting the blood were coated with methyl-chloro silane (Dri-Film). The blood was transferred to a tube similarly coated and centrifuged at 6000 rpm in an angle centrifuge for 10 minutes.

† Washed platelets obtained from 3 cc of hemophilic plasma by means of differential centrifugation were mixed with 1 cc of deplateletized plasma.

‡ To a mixture of 0.1 cc of 0.02 M CaCl_2 , 0.1 cc thromboplastin and 0.1 cc fresh oxalated plasma treated with $\text{Ca}_3(\text{PO}_4)_2$, 0.1 cc of serum was added.

TABLE II.
Evidence of a Substance (in the Blood of a Patient with a Hemophilia-like Condition) Which Inhibits the Activation of Thromboplastinogen.

	Coagulation time (Lee-White) min	Prothrombin activity remaining in serum 1½ hr after coagulation %
Normal blood	6	15 (32 sec)
Hemophilia-like blood	35	80+ (12 ")
Hemophilia-like blood, 1 vol., normal blood, 1 vol.	30	80+ (12 ")

to be activated. If it be a simple lack of thromboplastinogen, the addition of normal blood should restore the coagulation time approximately to normal and significantly increase the prothrombin consumption as is observed with hemophilic blood. This does not occur. In fact when one volume of the patient's blood is mixed with one volume of normal blood, the mixture has nearly as long a coagulation time as that of the patient's blood. The most obvious explanation is that this hemophilia-like blood contains an excess of a substance which inhibits the conversion of thromboplastinogen to active thromboplastin. This also explains why patients of this type are refractory to transfusions and to anti-hemophilic globulin. In uncomplicated hemophilia, the defect appears to be essentially a deficiency of thromboplastinogen, but the reports of Munro and Munro³ and of Lawrence and Craddock⁴ suggest that the disease can be complicated by the appearance in

the blood of an inhibitor. It is likely that the agent is the same as the one occurring in the patient of this report.

From these results it can be concluded that a thromboplastin deficiency may arise either from a lack of thromboplastinogen in the plasma or from an agent which inhibits the platelet factor. A third possible cause may be postulated, namely, a lack of the platelet factor. All three conditions are characterized by a markedly incomplete conversion of prothrombin.

Summary. The first step in coagulation is the conversion of thromboplastinogen to active thromboplastin by a platelet factor. The platelets from hemophilic blood react equally as well as those of normal blood. Evidence of a factor which inhibits the activation of thromboplastinogen has been found in the blood of a patient who has an acquired hemophilia-like condition.

⁴ Lawrence, J. S., and Craddock, C. G., *Science*, 1947, 106, 473.

³ Munro, F. L., and Munro, M. P., *J. Clin. Invest.*, 1946, 25, 814.

Dietary Impairment of Estrogen Response in the Immature Monkey.

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We have previously reported that the genital tract of immature chicks maintained on a diet deficient in folic acid shows only a slight growth response to estrogenic hormone.¹

The laboratory monkey (*Macacus rhesus*) is known to require exogenous folic acid, and the characteristic features of the deficiency syndrome have been described.^{2,3}

We wish to report that sexually immature monkeys which are maintained on a synthetic diet known to be deficient in folic acid fail to show the characteristic changes observed by Allen⁴ and others in estrogen-treated monkeys fed a natural diet.

Ten sexually immature female monkeys weighing between 2500 and 2900 g were individually caged and fed *ad libitum* a purified diet of the following percentile composition:⁵ C. P. dextrose, 73; vitamin-free casein (Sma-co), 18; salt mixture, 4; corn oil, 3; cod liver oil, 2.⁵ In addition, the following supplement suspended in 20 cc of water was offered each monkey daily: thiamine, 2 mg; nicotinic acid, 10 mg; riboflavin, 2 mg; pyridoxine, 2 mg; calcium pantothenate, 6 mg; choline, 100 mg; para-aminobenzoic acid, 200 mg; *i*-inositol, 200 mg; ascorbic acid, 50 mg; biotin, 50 μ g; dextrose, 2 g. When a monkey failed to consume the supplement voluntarily it was administered by stomach tube.

To the diet of 2 of the monkeys 5% by weight of whole dried liver substance was added for control.

The monkeys were observed for periods ranging from 43 to 66 days. As each animal on the experimental diet developed signs of deficiency characterized by apathy, muscular weakness, diarrhea, anorexia, weight loss and leukopenia, daily subcutaneous injections of 66 μ g of estradiol benzoate in 0.2 cc corn oil were administered for periods ranging from 5 to 10 days. Similar treatment was given the liver-supplemented animals after comparable periods of observation. Daily examination was made of the vaginal smear, the external genitalia, and the sexual skin.

The data are summarized in Table I. It will be seen that of 8 monkeys maintained on the deficient diet 6 failed to show any change in the external genitalia or sexual skin following estrogen treatment for 8 to 10 days. In the 2 liver-supplemented animals the characteristic external changes were readily observable by the end of the third day and were very marked by the end of the fifth day (Fig. 1 and 2). The vaginal smears in the non-responsive experimental animals showed little change following estrogen administration whereas the smears from the liver-supplemented animals showed the characteristic cornified cells.

These observations do not establish the specificity of the dietary deficiency produced

TABLE I.
Perineal Estrogen Response of "M" Deficient Monkeys.

Monkey	Days on diet	Days injected	Response
694	43	9	negative
675	53	8	"
19	60	10	"
18	60	10	"
B39	48	8	"
B18	48	8	"
B9	47	5	positive
46	48	5	"

Each monkey given 66 μ g (400 R.U.) of estradiol benzoate in 0.2 cc corn oil subcutaneously daily.

¹ Hertz, R., *Endocrinology*, 1945, **37**, 1.

² Day, P. L., Langston, W. C., and Darby, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 860.

³ Day, P. L., Nims, V., and Potter, J. R., *J. B. C.*, 1945, **161**, 45.

⁴ Allen, E., Hisaw, F. L., and Gardner, W. V., *Sex and Internal Secretions*, 2nd Edition, 1939, Chapter VIII.

⁵ Weisman, H. A., Rasmussen, A. F., Elvehjem, C. A., and Clark, P. F., *J. Nutrition*, 1945, **26**, 205.



FIG. 1.
Perineum of foliac acid-deficient monkey after 10 days estrogen treatment.

FIG. 2.
Perineum of control monkey after 5 days estrogen treatment.

since we have not attempted to restore the estrogen responsiveness in the foliac acid deficient monkey following the administration of crystalline foliac acid. However, we have observed such a restoration of estrogen response in the foliac acid deficient chick following the administration of synthetic foliac acid. Moreover, Day *et al.*² have shown that monkeys on diets comparable to that employed here show a good therapeutic response to foliac acid.

Monkeys are known to show wide variability in the length of time required for the development of foliac acid deficiency.² It is possible that the 2 animals on the deficient diet which showed a good response to estrogen were incompletely depleted of foliac acid at the time they were tested, although they were not grossly distinguishable from the others.

The mechanism and the biological signifi-

cance of the dependence of estrogen utilization on dietary foliac acid remain obscure. The existence of this relationship in two such widely diverse forms as the chick and the monkey suggests that the phenomenon may be of physiological significance. Perhaps the well-known sex difference in erythropoiesis may depend in some way upon this interrelationship between foliac acid and the estrogens. In this connection, Taber *et al.*⁶ have shown that the characteristic sex difference in red count in the chick may be reversed by administration of either male or female sex hormone. Analogous findings have been presented by Vollmer *et al.*⁷ for the rat.

⁶ Taber, E., Davis, D., and Domm, L., *Am. J. Phys.*, 1943, **138**, 479.

⁷ Vollmer, E. P., Gordon, A. S., Levenstein, I., and Charipper, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 409.

Summary. Of 8 sexually immature monkeys maintained on a folic acid-deficient, synthetic diet until evidence of dietary deficiency appeared, 6 failed to show the characteristic normal response to estrogen administration.

Two control monkeys fed the same diet plus liver and 2 of the apparently deficient animals showed typical estrogen response to similar hormone treatment.

16221 P

Renal Resistance in "Essential" Hypertension. Relation to the Effect of Sympathectomy on Blood Pressure.*

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Blood pressure and renal dynamics were studied preoperatively in 13 hypertensive patients subjected to extensive sympathectomy in 1940-42.^{1,2} These observations were repeated on 12 of these cases at intervals extending up to 8 to 40 months after operation. Current analysis of these data suggests that the preoperative renal resistance may indicate with reasonable accuracy whether a significant reduction in blood pressure will follow the operative procedure. While renal resistances were also calculated by the method of Lampport,³ a value for "crude" renal resistance is satisfactory. The "crude" renal resistance is defined as $\frac{\text{Blood Pressure}}{\text{Renal Blood Flow}} \times 100$.

The arithmetical mean of systolic and diastolic brachial arterial pressure determined by sphygmomanometer, is taken as the blood pressure; and the diodrast or para-amino hippurate clearance of blood in cc per minute per 1.73 square meters of body surface is considered to represent renal blood flow. The resistance value obtained was above normal in all cases. It was less than 22 in 5 cases with definite postoperative reduction in blood pressure and was 22 or more in 7 cases

with little or no reduction in blood pressure. The means of the postoperative diastolic blood pressure in these two groups were 88.8 and 120.3 mm Hg, respectively, the difference between these being significant (Fig. 1), when subjected to Fisher's method for the comparison of small samples.⁴ Diastolic pressures were averaged for each patient during periods of hospitalization for the studies. The pressures taken during the renal tests for each patient tend to be slightly higher than the corresponding hospital averages. Using either "test" diastolic, or $\frac{1}{2}$ (systolic plus diastolic) pressure values, the difference in the means of post-operative pressure in the two groups arbitrarily divided according to resistance, remains significant ($t = 5.41$, 4.49).

It is therefore unlikely that chance alone would produce the results obtained in this grouping. However, the data of our series alone is inadequate to prove that the difference in resistance is the only responsible factor. Several other factors may be involved. Four of the patients where blood pressure was markedly reduced by operation were the 4 youngest in the series. If we divide our patients according to age, a significant difference in the mean of postoperative pressure is noted when this division is made at or under 35 years (Fig. 1). The

* Aided by a grant from the Douglas Smith Foundation.

1 Adams, W., Alving, A. S., Sandiford, I., Grimson, K. S., and Scott, C., *Am. J. Physiol.* (Proceedings), 1941, 133, 190.

2 Grimson, K. S., Adams, W., Alving, A. S., and Landowne, M., in preparation.

3 Lampport, H., *J. Clin. Invest.*, 1943, 22, 461.

4 Fisher, R. A., *Statistical Methods for Research Workers*, 10th edition, G. E. Stechert, New York, p. 122, 1946.

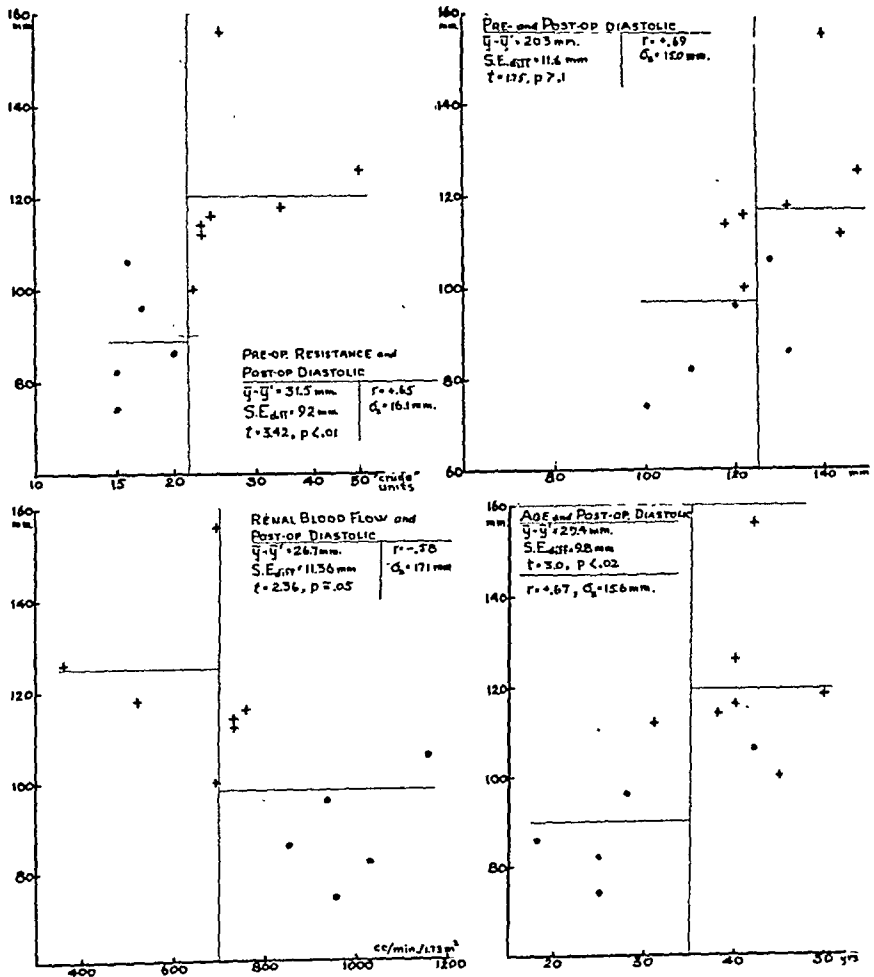


FIG. 1.

Scatter diagrams from data of 12 hypertensive patients subjected to sympathectomy. The averaged post-operative diastolic pressure in mm Hg (ordinates) is related to each of 4 pre-operative measurements. r indicates the first order coefficients for the data as plotted, and σ_y the standard error of the estimate. The means of ordinate values greater and less than arbitrary vertical divisions are indicated by horizontal lines. The difference of the means ($y-y'$), the standard error of this difference ($S.E._{diff}$), their ratio (t) and the probability value (p) are shown.

average preoperative "crude" resistance of the patients 35 years of age or younger was lower than the average of the patients over 35 years of age, but the difference has little significance ($p > 0.05$). Analysis of the component values used to derive "crude" resistance indicates that a grouping of cases on the basis of preoperative diastolic pressure level reveals no significant differences in results. Division according to preoperative renal blood flows at or below the value of

549 cc per minute selected by Foà *et al.*,⁵ or even at 700 cc per minute, does not result in a significant difference in the postoperative diastolic means. Only if the cases are divided at a renal blood flow of 800 cc per minute, are significant differences manifest. At this level the grouping is identical to that obtained by division at a "crude" resistance of 22 units.

⁵ Foà, P. P., Woods, W. W., Peet, M. M., and Foà, N. L., *Arch. Intern. Med.*, 1943, **71**, 357.

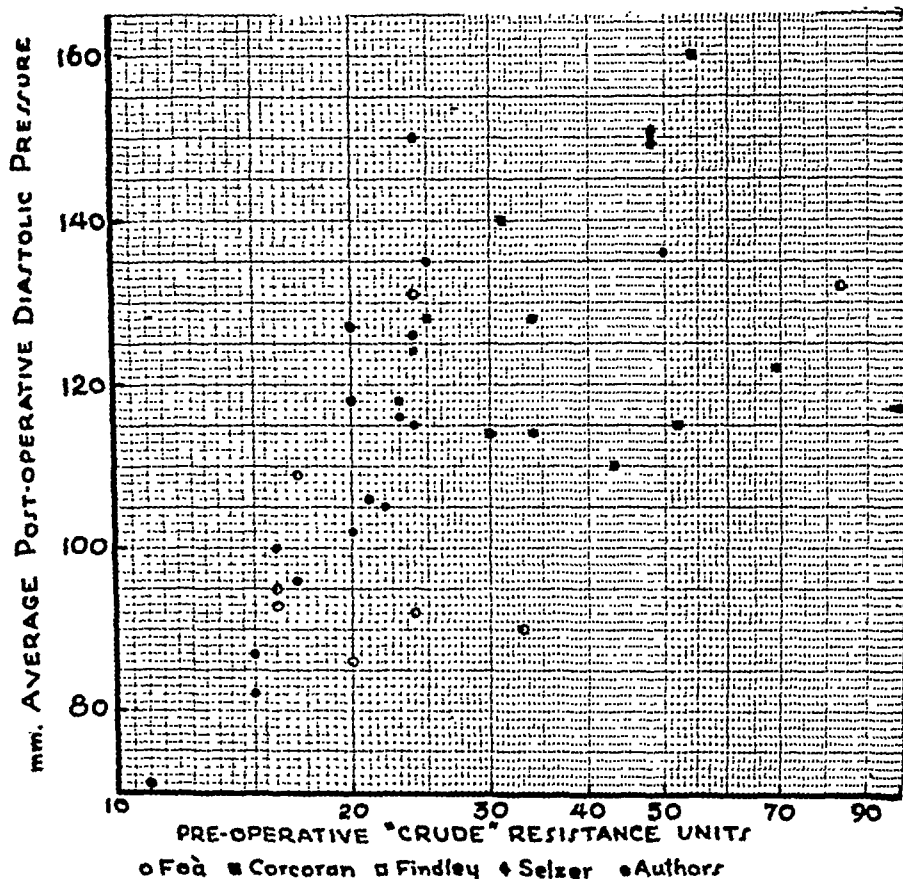


FIG. 2.

Composite scatter diagram relating preoperative renal resistance in "crude" units to the average postoperative diastolic pressure in mm Hg of 38 patients with hypertension subjected to sympathectomy. Twenty-six cases are calculated from data in the literature.⁵⁻⁸

Fig. 1 also indicates the zero order coefficients of correlation (r) and the standard error of the estimate (σ_e) of the relation of postoperative pressure to each of the 4 parameters mentioned, viz., log "crude" preoperative resistance, preoperative diastolic pressure, preoperative renal blood flow, and age. All show good correlation but with a wide scatter.

Fig. 2 indicates the relation between the postoperative diastolic pressure and the preoperative "crude" resistance, again for the present series (black dots) and for the calculated values in 26 additional cases reported in the literature.^{5,6,7,8} If these 26 cases are similarly divided into 2 groups on the basis of whether their calculated preoperative "crude" resistances are below 22 units, or are 22 or

more; the differences of the means of their postoperative diastolic pressures is also highly significant ($y - y' = 25$ mm, $p < 0.01$). No significant differences in postoperative diastolic means are revealed by groupings using age, preoperative diastolic pressures, or renal blood flows. If divided at a renal blood flow of 549 cc per minute, p is greater than 0.1, and, if the higher level of 700 cc per minute is used, the likelihood that chance explains the difference is almost as great

⁶ Corcoran, A. C., and Page, I. H., *Arch. Surg.*, 1941, **42**, 1072.

⁷ Selzer, A., and Friedman, M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 429.

⁸ Findley, T., Clinton, E., and Edwards, J. C., *Surgery*, 1942, **12**, 64.

($y - y' = 20.2$ mm, $p > 0.05$). Only 4 cases had preoperative renal blood flows above 800 cc per minute. Resistance values were calculated from the publications of these authors, using pressures at the time of the tests wherever designated by them, and without modification of data. Where hematocrit was not given, a value of 0.43 was assumed.

Conclusion. A relation of the preoperative renal resistance to the postoperative blood pressure has been demonstrated in patients with hypertension subjected to sympathectomy. A complete test of the reliability of this relation in predicting the result of sympathectomy requires additional data.

16222

Antagonism by Cellular Extracts of Effects of Respiratory Poisons on Onion Roots and Sea Urchin Eggs.

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Aqueous yeast extract has been shown to antagonize the deleterious effects of potassium cyanide, basic phenylmercuric nitrate and sodium azide upon respiration^{1,2,3,4} and of basic phenylmercuric nitrate on the growth^{1,5,6} of unicellular organisms and fragments of mammalian tissues. Study of the antagonism of yeast extract against the effects of these respiratory poisons has now been extended to intact multicellular structures, namely, the roots of the onion and the eggs of the sea urchin.

Onion Roots. Onions (*Allium cepa*) of approximately 5 cm in diameter, purchased in market, were germinated over water in the dark at room temperature. When the roots were approximately 4-7 cm in length they

were reduced in number to 10-25 of uniform appearance. The normal rate of growth was 1 cm to 1.5 cm per day. The poisons employed were basic phenylmercuric nitrate (PMN) in concentrations of 1:200,000 to 1:24,000 (7.5×10^{-6} to 6.3×10^{-5} M); potassium cyanide, 0.4% (0.06 M); mercuric chloride, 1:72,000 (5.1×10^{-5} M), and urethane, 3 and 5% (0.34 M and 0.56 M).

The crude yeast extract, in dilutions of 0.8 to 2.5%, was an alcoholic extract corresponding to Sample A as described by Cook, Kreke, and Nutini.⁷ In a few experiments beef spleen extract⁸ was used in concentrations of 0.4 and 0.2% with concentrations of 1.6×10^{-5} PMN. The onion roots were immersed about half their total length in the test solutions for 1 hour and were thoroughly rinsed before replacement in tap water. The lethal dose of the poison was established as the amount which would lead to the death of all of the roots as a consequence of 1 hour of exposure. On surviving roots, enlarged tips, if they developed, usually did so in the 24 hours following exposure to the test solutions.

¹ Cook, E. S., and Kreke, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 222.

² Cook, E. S., Kreke, C. W., Eilert, Sr. M. R., and Sawyer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 210.

³ Kreke, C. W., and Suter, Sr. M. St. A., *J. Biol. Chem.*, 1945, **160**, 105.

⁴ Kreke, C. W., and Suter, Sr. M. St. A., *Studies Inst. Divi Thomae*, 1945, **4**, 85.

⁵ Cook, E. S., and Kreke, C. W., *Nature*, 1940, **146**, 688.

⁶ Thomas, G. W., Fardon, J. C., Baker, S. L., and Cook, E. S., *J. Am. Pharm. Assn., Sci. Ed.*, 1945, **34**, 143.

⁷ Cook, E. S., Kreke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 23.

⁸ Staff, I. D. T., *Studies Inst. Divi Thomae*, 1947, **5**, 55.

TABLE I.

Gross Effects on Onion Roots After One Hour of Exposure to Respiratory Poisons and Yeast Extract.

Concentrations		Dead roots, %	Terminal swellings, %	Growth arrest, hr	Total growth 4 days, cm
Poison	Yeast extract, %				
0	0	0	0	0	4.6
Basic Phenylmercuric Nitrate.					
1:200,000	—	0	100	24	2.3
(7.5×10^{-6} M)	1.0	0	100	24	2.3
1:96,000	—	100	—	—	—
(1.6×10^{-5} M)	0.8	0	100	24	2.3
1:70,000	—	100	—	—	—
(2.1×10^{-5} M)	1.0	0	100	12-24	2.4
1:50,000	1.2	0	76	12-24	2.3
(3.0×10^{-5} M)	2.5	0	100	48*	1-1.5
1:48,000†	—	100	—	—	—
(3.1×10^{-5} M)	0.5	0	100	12-48	2.3
1:30,000	—	100	—	—	—
(5.0×10^{-5} M)	0.5	100	—	—	—
	1.0	80	20	72	1
1:24,000	—	100	—	—	—
(6.3×10^{-5} M)	1.0	100	—	—	—
	2.0	21	0	Indef.	?
	2.5	0	100	12-72	0.2-3.5
Potassium Cyanide.					
0.4%	—	100	—	—	—
(0.06 M)	1.0	0	0	Slight	3.5-4
Mercuric Chloride.					
1:72 000	—	100	—	—	—
(5.1×10^{-5} M)	1.0	0	0	Slight	4-4.5‡

* On'y 50% resumed growth.

† Only 30 minutes exposure.

‡ Secondary roots developed, after 5 days, from root tips and along entire immersed length.

The results are summarized in Table I. When the effects of the test solutions were sublethal, growth by elongation, in many instances was arrested in the succeeding 24-hour interval. During this time small swellings developed just behind the meristematic tip which were similar in appearance to those produced by exposure to colchicine and to various organic salts.⁹ Growth was usually resumed at a reduced rate on the second day and distal to the terminal swelling (Table I).

Yeast extract in no way modified the effects of a sublethal concentration of PMN, but prevented the lethal action of concentrations of the poison as high as 6.3×10^{-5} M, with the subsequent production of terminal swellings. In one experiment (PMN, 3×10^{-5} M, and yeast extract, 2.5%) only 50% of the roots resumed growth within 4 days. To determine if this was in part due to the high concentration of the yeast extract, onion roots

were exposed to a 2.5% concentration of the extract alone. In the next 4 days their average growth was only 5 cm while that of control roots on the same bulb which had been in water throughout, was 6 cm. By the 4th day the daily increment was equivalent to that of the control roots.

Against lethal amounts of KCN and HgCl_2 , a 1% concentration of yeast extract entirely protected the roots with no swellings or irregularities in form developing.

The action of urethane on onion roots differed from that of the PMN, KCN, and HgCl_2 . A 5% solution (0.56 M) acting for 1 hour was not lethal. Growth was arrested for 48 hours but no swellings developed. Exposure for as long as 6 hours to a 3% (0.34 M) solution was not lethal at the end of that time and elongation continued for the next 24 hours, but was arrested on the second day, during which time terminal swellings developed. By the 4th day the tiny meristematic tip distal to the swollen zone was dead.

⁹ Leván, A., *Nature*, 1945, 150, 751.

Twenty-four hours after exposure to 3% urethane and 1% yeast extract the roots were flaccid and dead in a zone 1 cm in length immediately behind the meristematic tip; no terminal swellings developed during the interval.

Two experiments with aqueous beef spleen extract in concentrations of 0.4 and 0.2% were made using 1:96,000 PMN (1.6×10^{-5} M), for an hour of exposure. With 0.4% beef spleen extract none of the roots was dead at the end of the exposure period. Growth was arrested for 24 hours, during which time 2 of the 12 roots developed small terminal swellings. Very slight growth (1-2 cm) resulted during the 5 days following exposure. With 0.2% spleen extract, 4 roots were dead at the end of 48 hours; the remaining 10 continued to grow very slowly, 2 developing swollen tips and the others becoming curved and irregular in form.

Sea Urchin Embryos. Fertilized eggs in the 2- to 4-cell stage of the sea urchin (*Tripleneustes esculentus*) were exposed for 1 hour to basic PMN and to mixtures of PMN and yeast extract and of PMN and beef spleen extract. Suspensions of fertilized eggs were prepared according to Just's method.¹⁰ About 10 ml of thick egg suspensions containing a few hundred eggs were diluted to 30 ml with fresh sea water and used for each experiment. In control experiments the developing eggs were ciliated motile blastulae within 20 hours. The concentrations of PMN used were 1:144,000 (1×10^{-5} M), 1:1.5 million (1×10^{-6} M), and 1:2 million (7.5×10^{-6} M); 1:3.2 million (4.7×10^{-7} M) and 1:4 million (3.8×10^{-7} M). The crude yeast extract was diluted to 0.5, 0.8, and 1.0%.

With all of the concentrations of PMN, except the most dilute solution, 95 to 100% of the eggs were dead at the end of 20 hours after exposure to the poison for 1 hour. Some of the eggs (43, 30 and 95%) resumed growth after removal from the more concentrated solutions of PMN (1×10^{-5} M, 1×10^{-6} M, 7.5×10^{-7} M), but only 2% of those in 1×10^{-6} M reached the blastula stage and

became motile. Most of the embryos treated divided but once more. Many of the embryos were anomalous in form and some of the cells cytolized. In the weakest dilution (3.8×10^{-7} M) the eggs were undamaged after 1 hour of exposure to the poison, resuming a normal rate of growth and development except for the slowing of the rate of absorption of the yolk.

Yeast extract (0.8%) did not protect the eggs against the lethal action of PMN 1×10^{-5} M, although a larger percentage resumed growth for a short period, after removal to seawater, than was the case with eggs exposed to PMN of the same concentration alone. None of the cells was cytolized. All of the eggs exposed to PMN 1×10^{-6} M and 1% yeast extract resumed growth, and half reached the motile blastula stage as contrasted with the 30% resuming growth, and 2% reaching the blastula stage after exposure to the poison alone. Mortality at 20 hours with yeast extract and the poison was 50% as compared to 95% without extract. There were also fewer anomalous forms and less cytolysis among the eggs exposed to the mixture of poison and yeast extract than among those exposed to the poison alone. At a PMN concentration of 4.7×10^{-7} M, yeast extract (0.8%) completely overcame the deleterious effects of PMN.

Evidence of a retarding action of the yeast extract itself lies in the observation that there was a more nearly normal rate of yolk absorption after exposure to PMN 3.8×10^{-7} M with 0.5% extract than with 1.0% of extract. Beef spleen extract at 0.8% concentration was less effective than yeast extract in antagonizing the deleterious effects of PMN on sea urchin eggs.

Discussion. It has been shown that, while basic phenylmercuric nitrate inhibits enzymes dependent upon the -SH group for their activity, it also inhibits iron porphyrin enzymes such as catalase and cytochrome oxidase,^{11,12,13} azide and cyanide likewise inhibit

¹⁰ Just, E. E., *Basic Methods on Eggs of Marine Animals*, Philadelphia, P. Blakiston's Son and Co., 1939.

¹¹ Cook, E. S., Kreke, C. W., McDevitt, Sr. M. of L., and Bartlett, Sr. M. D., *J. Biol. Chem.*, 1946, **162**, 43.

¹² Cook, E. S., and Perisutti, G., *J. Biol. Chem.*, 1947, **167**, 827.

the iron porphyrin enzymes.¹⁴ Yeast extract has accelerated the activity of cytochrome oxidase and offset the effects of PMN (Cook and Kreke, unpublished) and poisons specific for this enzyme.^{3,4} Moreover, yeast extract fails to antagonize poisoning of the respiration of yeast and mammalian tissue by urethane which inhibits the dehydrogenases.^{3,4} In the present work, the action of urethane on onion roots differed from that of cyanide and PMN in 2 respects, in that (a) the meristematic cells were first killed and (b) this effect could not be offset with the concentrations of yeast extract used.

The effects of respiratory poisons and yeast extract on multicellular structures appear to

¹³ Cook, E. S., Kreke, C. W., and Walsh, Sr. T. M., *J. Biol. Chem.*, 1946, **162**, 51.

¹⁴ McElroy, W. D., *Quart. Rev. Biol.*, 1947, **22**, 25.

resemble those described for unicellular organisms and mammalian tissue fragments. Cytologic studies are now in progress to determine if the toxic action is also evident in intracellular modifications. In these experiments reaction of the extracts with the poisons can not be excluded nor can possible direct effects of the extract on the enzyme systems or on the cell membranes. Further experiments will be undertaken to obtain information on these points.

Summary. The lethal and sub-lethal effects of basic phenylmercuric nitrate, potassium cyanide and mercuric chloride on onion roots and sea urchin embryos can be antagonized by yeast and beef spleen extract. These observations are in agreement with reports that yeast extract can offset the action of respiratory poisons on some enzyme systems.

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Determination of Serum Polysaccharides by the Tryptophane Reaction.*

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A number of investigators have described methods for the quantitative estimation of the polysaccharides associated with the serum proteins. Merten¹ estimated these bound sugars by determining the difference between the free sugar and the total reduction after acid hydrolysis of the serum. Lustig and Langer² applied the Tillman-Phillippi orcinol method to an alcohol precipitate of serum. Seibert and Atno³ adopted the carbazole reagent of Dische⁴ for the quantitative estimation of polysaccharide in serum, both directly on the proteins obtained by ethanolic precipitation and by difference between the reducing sugar and the total sugar determined with carbazole. Sheppard and Everett⁵ described a reaction for the colorimetric determination of carbohydrates by means of tryptophane. The latter determination has the advantage that tryptophane arising from the hydrolysis of protein obviously does not interfere with it. It is the purpose of this paper

to describe the application of this reaction to the rapid quantitative assay of non-glucosamine serum polysaccharides.

Experimental. Reagents: 1. Sulfuric acid solution, 77% by volume. 770 ml of C.P. concentrated sulfuric acid is added to 230 ml of distilled water.

2. Absolute ethanol.

3. Tryptophane solution, 1%. 1 g of *l*-tryptophane (Eastman Kodak) is dissolved in 99 ml of warm distilled water. The solution is filtered and stored in a glass stoppered bottle in the refrigerator until ready for use.

Method: The method for blood serum is as follows: The serum is diluted 1-2 with 0.9% sodium chloride. Two-tenths ml of the diluted serum is pipetted drop by drop into 10 ml of absolute ethanol in glass stoppered centrifuge tubes. The precipitate is centrifuged out and washed once with 10 ml of absolute ethanol. After centrifuging as before, the ethanol is decanted and the precipitate drained by inversion of the tube. One ml of distilled water and 7 ml of 77% sulfuric acid are then added, the tube is allowed to stand at room temperature for 10 minutes, to dissolve the precipitate, and it is then placed in an ice bath for 15 minutes. One ml of cold 1% aqueous tryptophane solution (filtered before using) is added to each tube without shaking. The contents of the tube are mixed and the tube placed immediately in a boiling water

* Aided by a grant from the Oklahoma Division of the American Cancer Society.

¹ Merten, Richard, *Biochem. Z.*, 1938, **297**, 304.

² Lustig, B., and Langer, A., *Biochem. Z.*, 1931, **242**, 320.

³ Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 1946, **163**, 511.

⁴ Dische, Z., *Biochem. Z.*, 1927, **189**, 77.

⁵ Sheppard, F., and Everett, M. R., *J. Biol. Chem.*, 1937, **110**, 1.

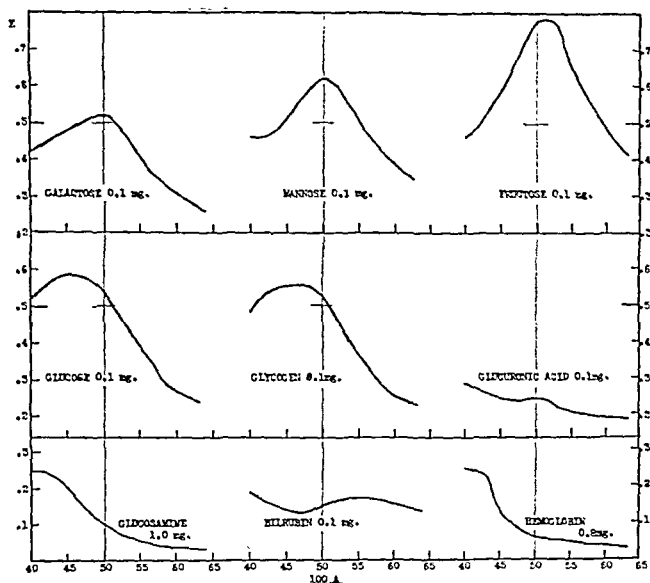


FIG. 1.

Absorption curves of single components after reaction with tryptophane.

bath for a period of 20 minutes. The tube is shaken once after it has been heated 10 minutes. After removing from the boiling water the tubes are cooled for 5 minutes in an ice bath and then allowed to stand 25 minutes at room temperature. The concentration is determined in a Coleman No. 11 spectrophotometer by reading against a blank (without the serum precipitate) which is run through the above procedure.

Standard: Equal amounts of *d*-galactose and *d*-mannose were used as a standard since present evidence indicates that the polysaccharide of serum is composed of equimolecular amounts of glucosamine, mannose, and galactose.⁶ As shown by absorption curves (Fig. 2) glucosamine has little influence on readings made at 500 $m\mu$, increasing the absorption only 1%.

Results. Absorption Curves. Absorption curves for mannose, galactose, glucose, glycogen, fructose, glucosamine, glucuronic acid, bilirubin, and hemoglobin are shown in Fig. 1. The maximum absorption for galactose and for mannose is at 500 $m\mu$, for fructose at 520 $m\mu$, for glucose at 460 $m\mu$. As expected.

glucose and glycogen produce similar curves.

Absorption curves of mixed sugars are shown in Fig. 2. The optical densities of the individual constituents are additive within experimental error. A typical serum absorption curve, also absorption curves for ascitic fluid, and hydrocele fluid are included in Fig. 2. All curves have been calculated to the same height at 500 $m\mu$. It is noteworthy that the serum, ascitic fluid, and hydrocele fluid curves differ from the galactose-mannose-glucosamine curve by their greater absorption in the 400-480 $m\mu$ range. However, absorption curves of partially purified serum polysaccharide (polysaccharide: total nitrogen ratio = 0.97 : 1) and a similar polysaccharide from hydrocele fluid (polysaccharide: total nitrogen ratio = 0.33 : 1) resemble closely the standard galactose-mannose-glucosamine curve. The enhanced absorption between 400 and 480 $m\mu$ is therefore due to the serum protein.

When the above described procedure is followed, except that 1 ml of distilled water is added to the serum precipitate in place of the tryptophane solution and the result is read against a blank containing 80 γ of tryptophane (the amount present in 0.0667 ml of

⁶ Meyer, K., *Adv. in Protein Chemistry*, 1945, 11, 249.

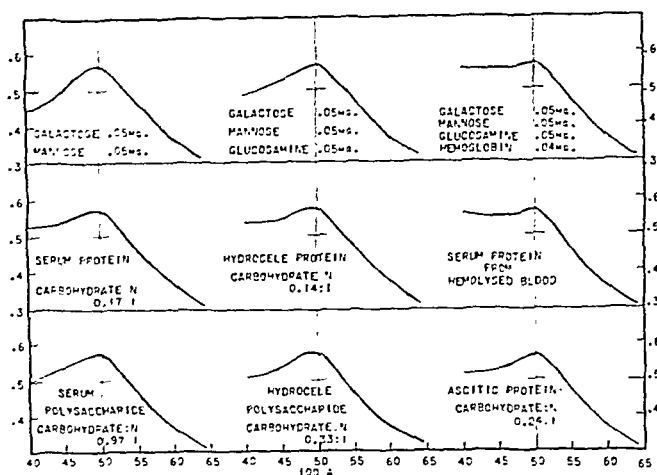


FIG. 2.

Absorption curves of sugar mixtures and of body fluids after reaction with tryptophane.

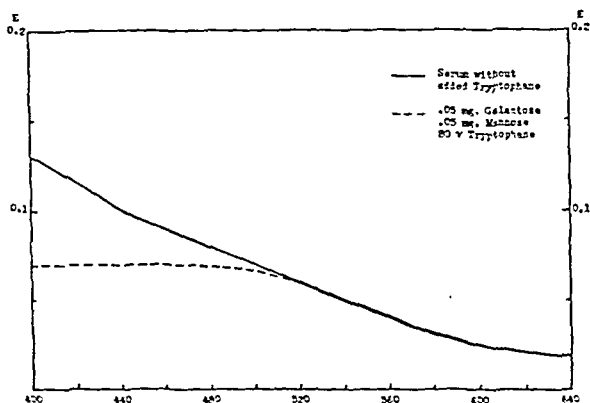


FIG. 3.

Absorption curve of serum protein without added tryptophane read against 60% sulfuric acid.

serum containing 7% protein) the upper absorption curve in Fig. 3 is obtained. The lower curve was obtained by allowing a standard solution of galactose, mannose, and glucosamine to react with 80 γ tryptophane under the above conditions. The difference between the two curves should then represent the result of chromophoric reactions of the system other than the tryptophane-sugar reaction. When this derived curve is used to correct the serum absorption curve, they approach the absorption curves of the galactose-mannose-glucosamine mixtures within experimental error. It is noteworthy that no appre-

ciable correction is necessary for the absorption at 500 $m\mu$. The nature of the substance or substances in serum protein which increase absorption in the region between 400 and 480 $m\mu$ was not discovered. Addition to standard sugar solutions of tyrosine, cystine, and histidine at levels found in serum protein failed to influence the shape of the galactose-mannose-glucosamine curve.

Hemolysis of the blood sample, if extensive, results in a positive error; the effect of hemoglobin on the absorption curve (Fig. 2) is most striking in the 400-480 $m\mu$ range and small at 500 $m\mu$. Therefore, the polysac-

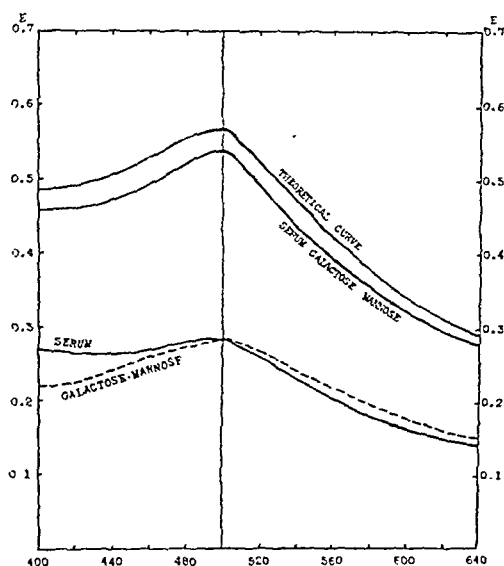


FIG. 4.

Effect of added sugar upon serum-tryptophane absorption curve.

solutions of the precipitates of several serums using the Folin-Wu procedure with a Somogyi (zinc hydroxide) precipitation. No glucose was found in any of these precipitates.

To determine the completeness of the polysaccharide precipitation the total carbohydrate and the polysaccharide were determined by the tryptophane method on the same samples of serum. Free sugar was also determined on these serums by the Folin-Wu procedure using the Somogyi precipitation. When the difference in absorption between the total carbohydrate and the polysaccharides was calculated as glucose, the results are as follows:

	Serum 1	Serum 2
Polysaccharide by tryptophane method	132	118
Glucose by Folin-Wu method	97	102
Total carbohydrate (calculated)	229	220
Total carbohydrate by tryptophane method	228	224

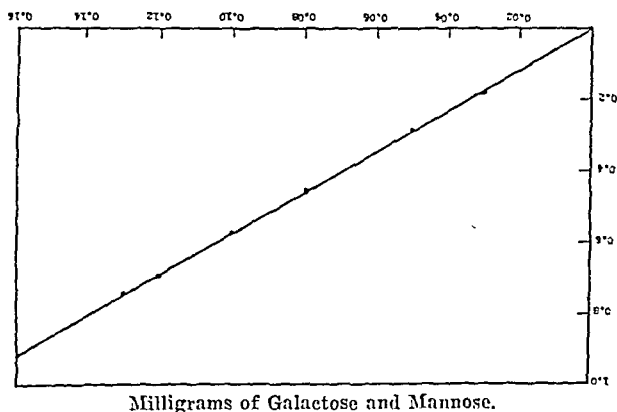


FIG. 5.

Concentration curve of the sugar-tryptophane system at 500 $m\mu$.

charide of slightly hemolysed samples may be determined with reasonable accuracy.

Recovery Curves. Mixtures of standard galactose and mannose with serum gave the absorption curve shown in Fig. 4. The height of this curve varies between 94% and 97% of the calculated value obtained by adding the absorption curve for the sugars to that of the serum.

Efficiency of the Precipitation Process. Since it was necessary to learn if any free sugar is carried down in the ethanolic precipitation, glucose was determined on aqueous

Since the total carbohydrate figures determined directly or by calculation agree quite closely and since the precipitate contains no glucose, the serum polysaccharides are apparently completely precipitated by the procedure described.

Calculations. In the concentration range, 0.02 mg to 0.16 mg of an equimolecular mixture of galactose and mannose, the Lambert-Beers law applies, as indicated in Fig. 5. Calculation of unknown concentrations may be made by either reading from the concentration curve or by the following formula:

$$\frac{\text{Ex}}{\text{Es}} \times .1 \times \frac{100}{.0667} = \text{mg\% polysaccharide as galactose and mannose}$$

where Ex is the extinction (log 1/transmission) of the unknown, and Es is the extinction of 0.1 mg of an equimolecular mixture of galactose and mannose.

Effect of Aging on Absorption Curves. When the solutions were read at various times after development of color, absorption curves similar to those shown in Fig. 6 were obtained. During the first 30 minutes, absorption increased in the 400-480 $m\mu$ range. No significant change in the curve occurred between 30 and 60 minutes. Consequently, for quantitative results the samples were read between 30 and 60 minutes after removal from the hot water bath.

Effect of Heating Time on Color Development. When the heating time in the boiling water bath was varied between 10 and 30 minutes, the absorption curves shown in Fig. 7 resulted. There is a progressive increase in the absorption at both ends of the curve as the heating time is increased. Little variation occurred at 500 $m\mu$ except in the 25-minute curve for the galactose-mannose mixture.

Quantitative Results on Human Sera. The

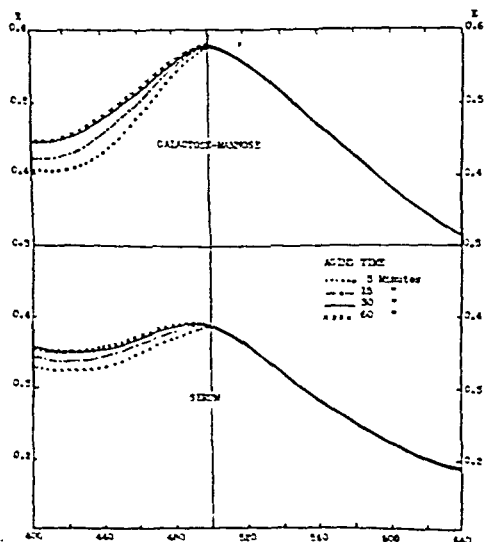


FIG. 6.

The effect of aging after removal from the boiling water bath upon the absorption of the sugar-tryptophane system.

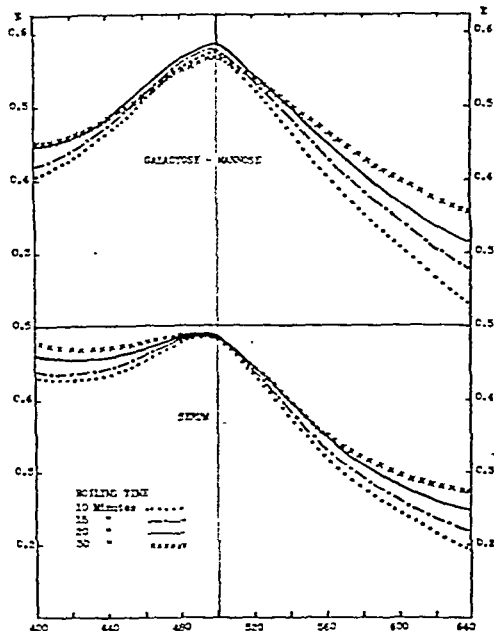


FIG. 7.

The effect of heating time on the absorption of the sugar-tryptophane system.

concentrations of polysaccharide, exclusive of glucosamine, in the sera of 59 normal persons representing both sexes and all ages varied between 80 mg % (fetal blood) and 139 mg % (blood from a senile man). These values are in general agreement with those of Siebert.³ Detailed results from studies of normal and pathological subjects will be given in another paper. The method described has considerable accuracy, the average difference between duplicates being 2.4% in a large number of blood serum samples.

Summary. A colorimetric method based on the reaction of tryptophane with carbohydrates is described for the determination of serum non-glucosamine polysaccharide. The absorption maximum for the carbohydrate-tryptophane compound is at 500 $m\mu$ for galactose and for mannose, 460 $m\mu$ for glucose, and 520 $m\mu$ for fructose. Glucuronic acid, glucosamine, and hemoglobin have maxima at wave lengths below 400 $m\mu$. Glucuronic acid also exhibits a weak band at 480-500 $m\mu$.

The absorption curves for alcohol precipitates from serum, ascitic fluid, and hydrocele fluid differ from postulated galactose-mannose-

glucosamine curves by showing greater absorption in the 400-480 $m\mu$ range. This difference diminishes when the polysaccharide is partly freed from protein.

Hemolysis, if marked, introduces a positive

error in the method. The color produced by the carbohydrate tryptophane reaction obeys the Lambert-Beers law within the limits investigated.

16224

Oxygen Uptake of Human Placental Tissue as Affected by Selected Substrates and Drugs.*

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Very few human tissues other than the placenta are readily available for *in vitro* studies. Despite this fact, little is known about the influence of metabolites or of pharmacologic agents upon this tissue. Any knowledge that may be gained about the behavior of this complex organ, furthermore, may assist in understanding some unsolved problems of human reproduction. We have selected for study the substrates commonly used for the *in vitro* study of tissue metabolism, and those drugs most frequently employed in obstetrics.

Early reports upon the oxygen uptake of human placental tissue¹⁻⁴ gave very low Q_{O_2} values, perhaps attributable to their methods. Loesser⁵ studied the oxygen consumption, anaerobic and aerobic glycolysis and in placenta two months of age found values for Q_{O_2} of 5.0, $Q_L^{N_2}$ of 7.0 and $Q_L^{O_2}$ of 5.1. At term, these values were 1.4, 3.5, and 0.57, respectively. Wang and Hellman⁶ demonstrated

changes in placental Q_{O_2} with relation to age in a manner corresponding well with the histologic changes in the villi. The Q_{O_2} was found to decrease from 5.3 at the second month to 1.7 at term, and the addition of glucose to the medium did not alter this value. No reports have been found regarding the influence of any other agents upon this tissue.

Methods. Placentas were placed in an ice-packed container immediately following delivery. The tissue was prepared for respiration studies in a cold box at 5°C.⁷ In choosing the experimental material necrotic, highly calcified, or traumatized areas were avoided. Thick sagittal slices were immersed in Ringer's solution and the villi teased free with dissecting needles. Samples of villi weighing from 200 to 250 mg were blotted on filter paper, weighed, and transferred to Warburg flasks. Conventional manometric methods were used throughout these studies. The medium consisted of 2.0 ml of Krebs-Ringer-phosphate solution (pH 7.3) modified to conform more closely to the composition of interstitial fluid.⁸ The substrate was 0.2% glucose unless otherwise specified, and the gas phase was oxygen. The oxygen consumption is expressed as microliters of oxygen per milligram dry weight tissue per hour (Q_{O_2}). In testing the effect of drugs upon oxygen uptake, a control period of 80 minutes preceded the experimental

* Aided by grants from the John and Mary Markle Foundation, New York City, and the National Institute of Health, Bethesda, Md.

¹ Reeh, W., *Z. f. Biol.*, 1924, **80**, 231.

² Kustner, H., and Siedentopf, H., *Arch. Gynak.*, 1929, **138**, 131.

³ Adler, K., *Arch. Gynak.*, 1930, **140**, 338.

⁴ Matsushita, D. T., *Mitt. a. d. med. Akad. zu Kyoto*, 1935, **13**, 872.

⁵ Loesser, A., *Arch. Gynak.*, 1932, **148**, 118, and **148**, 123.

⁶ Wang, H. W., and Hellman, L. M., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 31.

⁷ Fuhrman, F. A., and Field, J., II, *J. Biol. Chem.*, 1944, **153**, 515.

⁸ Elliott, H. W., Warrens, A. E., and James, H. P., *J. Pharm.*, 1947, **91**, 98.

period of 80 minutes, with readings obtained at 20-minute intervals. The "normal" \dot{Q}_{O_2} for each placenta is the average \dot{Q}_{O_2} of all flasks for the first 80 minutes.

Results. The substrates studied were dextrose, sodium pyruvate, sodium succinate, and hydroquinone. The mean \dot{Q}_{O_2} of 29 placentas delivered within 2 weeks of the estimated date of confinement was found to be $1.92 \pm$ a standard deviation of 0.373. The presence of dextrose (0.011 M) made no significant difference in the rate of oxygen uptake. The \dot{Q}_{O_2} could be maintained for 5 hours with a decline of only 13%. Similar results were obtained with sodium pyruvate (0.045 M). The addition of sodium succinate (0.017 M), however, gives an initial \dot{Q}_{O_2} of 3.6, with a decline of 34% in 2 hours and 66% in 4 hours. Increasing the succinate concentration to 0.2 M raised the initial \dot{Q}_{O_2} to 7.3, with a decline of 23% in 2 hours. With hydroquinone in concentrations of 0.045 M, 0.091 M, 0.182 M, and 0.364 M, the initial \dot{Q}_{O_2} values were 16.4, 20.8, and 25.4 respectively. In 2 hours, all 4 values had fallen 78%. For any substrate studied, the figures represent the means of at least 6 observations on 3 placentas. While both succinate and hydroquinone are oxidizable substrates, the maximal \dot{Q}_{O_2} obtainable with the former is limited by the concentration of enzymes in the succinoxidase system, whereas the maximal rate obtainable with hydroquinone (after correction for auto-oxidation) is limited only by the cytochrome-cytochrome oxidase system.

The drugs selected for study were merperidine (Demerol), methadon (10820, amidone, Dolophine), morphine, Amytal, and scopolamine. Observations were also made on diethylstilbestrol and dinitrophenol. Their effects upon the oxygen uptake of placental tissue are summarized in tabular form.

It will be noted that merperidine, Amytal, and scopolamine have only a depressant action, whereas methadon has a stimulating action in the lower concentrations. Morphine has no influence upon oxygen uptake. Dinitrophenol has the usual marked stimulating effect, while diethylstilbestrol, in aqueous suspension, has the same depressant effect noted on homogenates of liver and pituitary⁹ or upon cellular

suspensions of rat brain.¹⁰

Discussion. These studies indicate that the behavior of placental tissue in the Warburg flask is similar in many respects to other mammalian tissues. Like muscle or kidney, placental tissues contain stored substrates, since neither glucose nor pyruvate increase the \dot{Q}_{O_2} above the non-substrate value. In common with most tissues, placental \dot{Q}_{O_2} in the presence of succinate or hydroquinone is dependent, within limits, upon substrate concentration and is much higher than that obtained with such carbohydrate substrates as glucose or pyruvate.

The actions of the drugs tested agree qualitatively with effects reported for brain and other tissues. If the mechanism of drug action is likewise similar, it may be assumed that merperidine and methadon inhibit respiration by their action on certain dehydrogenases involved in the oxidation of carbohydrates, whereas Amytal exerts its inhibitory action at a point between the flavoproteins and cytochromes. Comparisons of placenta with other tissues may be made by reference to the remarks in Table I.

With the exception of Amytal, it will be noted that the concentrations of drugs employed are far in excess of the probable therapeutic concentration. It would not be anticipated, therefore, that the employment of such agents in the usual amounts would affect the total oxygen consumption of the placenta *in vivo*. It should be pointed out, furthermore, that the influence of a drug upon placental respiration is not related to the influence which that same drug may have upon the respiratory centers of the fetus. Finally, it must be recognized that our present state of knowledge does not permit us to surmise what effects, if any, a stimulation, whether by succinate or dinitrophenol administration, or a moderate drug depression of oxygen uptake would have upon the multiple functions of placental transfer and secretion.

Summary. The rate of oxygen consumption of fresh human chorionic villi was meas-

⁹ McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, 9, 165.

¹⁰ Gordan, G. S., and Elliott, H. W., *Endocrin.*, 1947, 41, 517.

TABLE I.
Effect of Drugs upon Oxygen Uptake of Human Placental Tissue *in vitro*.

Drug	<i>In vivo</i> conc.*	<i>In vitro</i> conc.	Maximal effect on Q_{O_2} (in % change from control value)	Comparison with other tissues
Morphine	2×10^{-6} M	2.5×10^{-4} M 5.0×10^{-3} M	0 0	As found for brain slices. ⁸
Merperidine	1.8×10^{-5} M	2.5×10^{-4} M 5×10^{-4} M 1×10^{-3} M 7.5×10^{-3} M	-10 -27 -43 -93	Approximately 10 times as sensitive to merperidine as brain slices. ⁸
Methadon	2×10^{-6} M	2.5×10^{-4} M 5×10^{-4} M 1×10^{-3} M 2×10^{-3} M	+22 +38 +12 -93	Post stimulatory inhibition at low concentrations not as marked as with brain slices. ⁸ Similar to diaphragm. ¹¹
Scopolamine	8×10^{-8} M	2.5×10^{-4} M 5×10^{-3} M 2×10^{-2} M	-11 -21 -56	Initial inhibition followed by partial recovery after 80 minutes. [†]
Amytal	5×10^{-5} M	1×10^{-4} M 2×10^{-4} M 4.5×10^{-4} M 9×10^{-4} M 1.8×10^{-3} M	0 -9 -31 -63 -97	Similar to effects of Amytal on brain slices. ¹²
Dinitrophenol	—	4×10^{-6} M	+56	Usual effect of dinitrophenol.
Stilbestrol	—	aq. suspension 4 mg./vessel	-88	As found for brain homogenates. ¹⁰

* Estimated extracellular concentration after therapeutic dose.

† Data for tissues other than human placenta not available.

ured by conventional manometric methods. The addition of dextrose or pyruvate did not influence the Q_{O_2} whereas succinate and hydroquinone increased the rate in proportion to their concentrations.

The Q_{O_2} of human placental tissue is depressed by merperidine, Amytal, scopolamine and diethylstilbestrol and stimulated by dinitrophenol. Morphine had no effect, while

methadon stimulated respiration in low concentrations and depressed it at higher levels. None of the drugs exerted an effect in therapeutic concentrations.

¹¹ Bachelor, A. L., and Elliott, H. W., *Fed. Proc.*, 1948, **7**, 203.

¹² Fuhrman, F. A., and Field, J., II, *J. Pharmacol.*, 1943, **77**, 392.

Quantitative Distribution of an Esterase Among Cytoplasmic Components of Mouse Liver Cells.*

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It was previously demonstrated by Dounce¹ that nuclei of rat liver cells, isolated by differential centrifugation of cell suspensions treated with citric acid, contained a concentration of esterase which was about 50% of that found in the whole tissue; methyl butyrate was used as the substrate. If the ratio of nuclear to cell volume is taken to be 6:100 for hepatic cells, as approximated by Marshak,² then the separated nuclei contain only around 3% of the esterase activity of the cell. It is therefore apparent that a study of the cytoplasmic components of the liver cell is essential to an understanding of the intracellular localization of the esterase. The unusually high concentration of esterase in the liver lends interest to an investigation of this nature since the special accumulation of the enzyme in this tissue can hardly be considered a merely fortuitous circumstance. Just which of the many esterifications and de-esterifications known to take place in the liver are catalyzed by this enzyme still remains to be elucidated. The distinct differences in the nature and properties of liver esterase and lipase have been known for some time.³

The quantitation of the activities observed in the various separated fractions as related to the activity of the whole cell has been emphasized in the present investigation since, as Danielli⁴ has pointed out, the centrifugal segregation of cellular components may result in an alteration of the enzymatic activities as they exist *in situ* through separation of

naturally occurring inhibitors, activators, etc.

Experimental. Livers from 2 or 3 female ZAF₁ strain mice were used in each experiment. The portal vein was clamped prior to the removal of the organ, ether anesthesia being used throughout. In the subsequent procedures up to the point of the determination of enzyme activity, care was taken to maintain the tissue as close to 0°C as possible.

The whole livers were put through a tissue press and weighed. The material was transferred to a mortar and ground thoroughly for 4 minutes with the slow addition of 4 volumes of alkalized physiological saline solution (2 cc of 0.1N NaOH per liter of 0.85% NaCl). The diluted tissue sample was further ground in a Potter-Elvehjem homogenizer⁵ for about 20 seconds. This ground tissue preparation was assumed to be a 5 times dilution of the original tissue sample.

The differential centrifugation procedure employed was essentially that of Claude⁶ with some modifications and additions and is summarized in Fig. 1. The washings indicated in the figure were carried out with volumes of physiological saline solution equal to that of the supernate in each case. The values of (g) were calculated from the center of the centrifuge tube in every instance. The time for the whole separation procedure from the initial removal of the liver was approximately 3 hours. Esterase activity was determined by a manometric method similar to that of Rona and Lasnitzki⁷ in a Warburg apparatus. 0.5 cc of the enzyme solution was placed in the sidearm of the reaction flask and 1.5 cc of

* Aided by a grant from the Medical Research Fund of the Graduate School of the University of Minnesota.

¹ Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

² Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 575.

³ Sobotka, H., and Glick, D., *J. Biol. Chem.*, 1934, **105**, 199.

⁴ Danielli, J. F., *Nature*, 1946, **157**, 755.

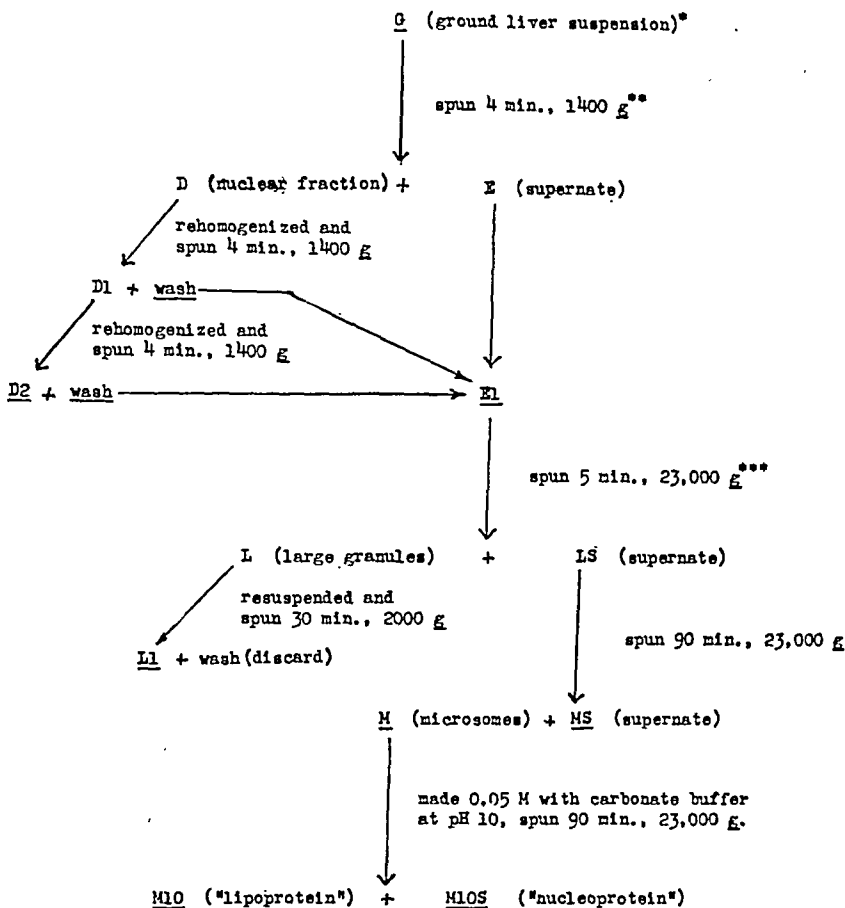
⁵ Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

⁶ Claude, A., *J. Exp. Med.*, 1946, **84**, 51.

⁷ Rona, P., and Lasnitzki, A., *Biochem. Z.*, 1924, **152**, 504.

CYTOPLASMIC DISTRIBUTION OF LIVER ESTERASE

FIGURE 1. FRACTIONATION OF LIVER CELL SUSPENSION BY DIFFERENTIAL CENTRIFUGATION



* Ground 4 min. in mortar with 4 volumes of alkalized physiological saline solution, then ground for 20 seconds in Potter-Elvehjem homogenizer.

** Servall angle centrifuge operated in refrigerator.

*** Multi-speed attachment to International centrifuge operated in cold room at -15°C .

bicarbonate-Ringer solution was introduced into the main chamber of the vessel. 0.022 cc of methyl butyrate was pipetted into the main chamber; the final concentration of 1% of the substrate in the complete reaction mixture thus obtained gives the optimum rate of hydrolysis without excess-substrate inhibition. The control vessel contained 0.5 cc of bicarbonate-Ringer solution in the sidearm in place of the enzyme solution. A 5% CO_2 , 95% N_2 gas mixture was passed through the system before the contents were mixed.

Nitrogen determinations were conducted by the Pregl micro-Kjeldahl method.

Results. The results are shown in Tables I and II. The unit of enzyme activity was chosen as the amount of enzyme which liberates 1 μl of CO_2 per hour at pH 7.4, 37.5°C and at the optimum substrate concentration. In the initial separation of the D and E fractions the cytoplasm was shown to contain most of the esterase of the cell. The N content is about 2.5 times, the enzyme activity per g of wet liver tissue is almost 5 times,

TABLE I.
Esterase Distribution Among Fractions of Mouse Liver Cell.

Exp.	1				2				3			
	N yield, % of G		Enz. conc., units/ μ g N		Enz. yield, % of G		Enz. conc., units/ μ g N		N yield, % of G		Enz. yield, % of G	
Fraction												
G	100		16.3	100	100		13.8	100	100		10.4	100
D ²	29		7.6	14	27		9.4	19	26		6.8	17
E1	64		20.2	80	67		13.5	65	70		12.6	85
L1	11		23.2	15	10		23.1	17	12		17.5	20
M					10		52.9	39	12		47.0	55
MS					42		3.8	12	46		3.3	15
M10									6		55.0	34
M10S									6		19.0	10

Note: N content of E and G were found to be 2.2% and 3.8%, respectively, based on wet weight of liver tissue.

TABLE II.
Summation of Nitrogen and Enzyme contents of Various Fractions.

Exp.	Nitrogen										Enzyme			
	D2 + E1		L1 + M + MS		M10 + M10S		D2 + E1		L1 + M + MS		M10 + M10S			
	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory
1	93	100					92	100						
2	94	100	62	67			84	100	68	65				
3	96	100	70	70	12	12	102	100	90	85	44			55

and the enzyme activity per μg N is around 2 times as great in the E fraction as in the D2. From the study of the N and enzyme distribution among the particulate fractions of the cytoplasm, Table I, it is clear that, although the greatest part of the N was confined to the supernate, MS, from the microsome separation, the largest portion of the enzyme was contained in the microsome fraction, M.

It has been shown that the separation of the M fraction into "lipoprotein" (M10) and "nucleoprotein" (M10S) fractions can be effected by treatment with a carbonate buffer at pH 10.⁸ The separation of the M into M10 and M10S fractions resulted in an equal distribution of N, however, the enzyme was concentrated in the M10 portion. These results were confirmed in duplicate experiments.

Examination of the data reveals that the separation of the various particulate fractions does not result in a noticeable loss of enzyme activity. This is shown by totaling the N values and enzyme activities found in the various sub-fractions and comparing the sums with the theoretically expected values. For example, the sum of the N values found in the L1, M and MS fractions should approximate the observed E value. These comparisons are summarized in Table II. The fact that the summation of enzyme activities approximated the theoretical values indicates that naturally occurring activators or inhibitors of the esterase in the mouse liver cell, if present, were not separated from the enzyme by the fractionation procedures.

It is possible that the presence of considerable activity in the L1 fraction and some activity in the D2 fraction may be derived from contamination of these fractions with microsomes. Electron microscopic examinations of the L1 fraction in other comparable

experiments appear to lend weight to this possibility. The presence of some intact cells is another possible source of the activity in the D2 fraction. The esterase activity found in our nuclear fraction (D2) therefore cannot be compared with Dounce's¹ values for isolated hepatic nuclei.

The isolation of pure nuclei with 1% citric acid, essentially the method of Marshak,² was carried out in one experiment, but the enzyme activity found was negligible. The esterase concentration in serum was also determined and was found to be less than 1% of that in the liver tissue. Hence no appreciable activity could be ascribed to contamination with serum.

Summary. The esterase in the cytoplasm of mouse liver cells was found to be localized predominantly in the microsome fraction which contained an average of 47% of the enzyme in the whole tissue and 63% of that in the entire cytoplasm. Other cell fractions, the nuclear material, the large cytoplasmic granules, and the supernate from the microsome separation contained an average of 17%, 17%, and 14%, respectively, of the enzyme in the whole tissue. Enzyme activity per μg of N was greatest in the microsomes which contained a concentration that was 4.2 times that of the whole tissue. In the other particulate fractions, the nuclear material, the large cytoplasmic granules, and the supernate from the microsome separation, the enzyme concentration was found to be 0.6, 1.6, and 0.3 times, respectively, that of the whole tissue. The "lipoprotein" fraction of the microsomes displayed about 3 times the enzyme concentration found in the "nucleoprotein" fraction. Summation of the enzyme content of the substituent fractions indicated no appreciable loss during the separation procedures.

We are indebted to Dr. J. J. Bittner for the mice used in these experiments.

⁸ Barnum, C. P., and Huseby, R. A., unpublished data.

Lipids of the Fasting Mouse. IV. Liver Total Lipid Content.

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The inter-relation of liver and muscle in the complex mechanisms of fat utilization have often been considered; the fasting mouse has proved to be a valuable object of study in this connection.^{1,2} Despite the rapid mobilization of available carcass fat and its prompt metabolism in the first days of fasting, the liver weight decreases in a regular fashion so that approximately half its initial weight is lost during a four day fast. The liver total lipid in this period exhibits a striking fatty accumulation followed by a reduction to below normal values.

In earlier studies, considerable variation had been observed in mortality, in liver weight loss and in accumulation of total lipid in the liver. Thus, a mortality of 66% was reached in 4 fasting days when the room temperature was occasionally permitted to fall even from 25° to 20° C, whereas only 22% mortality had occurred by the same day when the room temperature was held at 25° C. In the case of liver weight, the losses had been sufficiently irregular so that no simple tendency could be described. As to the liver total lipid, in one study the amount present had approximately doubled after 1 day of fasting but thereafter decreased to less than normal amounts; however, with more careful room temperature control and longer survival, the liver total lipid content had remained elevated on both the second and third fasting days. Finally, it was desired to extend the period of observation from 4 to 5 fasting days. With these several problems in mind, the following experiment was undertaken.

¹ Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Stoneburg, C. S., Oleson, M. C., and Whitehead, R., *J. Biol. Chem.*, 1941, **130**, 597.

² MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D., *J. Biol. Chem.*, 1942, **143**, 473.

Experimental. The fasting procedure in general was as described before.¹ Three months old, male, albino mice previously maintained on a diet of oats and Purina dog chow were fasted in individual cages. Water was supplied *ad lib.* and the room temperature was kept at 25°C. The mice were lightly anesthetized with ether and sacrificed by bleeding from the axillary artery.³ The livers were removed quickly and analyzed by standard procedures for total lipid.⁴

Mortality. No mice died during the first 2 fasting days; thereafter the mortality rapidly increased to about 50% on the fifth fasting day (see Table I). The temperature was maintained at 25°C in the animal room; at this temperature, the results reflected a mortality rate similar to that previously seen. A lower room temperature is known to be less favorable to survival.

Body Weight Loss. The average initial body weight varied from 20 to 22 g; the extreme variation among individual mice was 19 to 24 g. The body weight when recorded at the time of sacrifice was calculated as the sum of the carcass weight, the liver weight, and the weight of the blood sample. An almost linear decrease in body weight was observed with fasting. The average percentage body weight losses for the 2 preceding series^{1,2} and for the current experiments were almost identical. The average mouse lost about a third of its original weight

TABLE I.
Percentage Mortality: The Relation to Room Temperature Control.

Room temperature °C	Days of fasting				
	1	2	3	4	5
20-25	0	13	39	66	— (1)
25	0	0	0	22	— (2)
25	0	0	10	55	50 (current)

³ Kuhn, L. R., *Science*, 1941, **93**, 504.

⁴ Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.

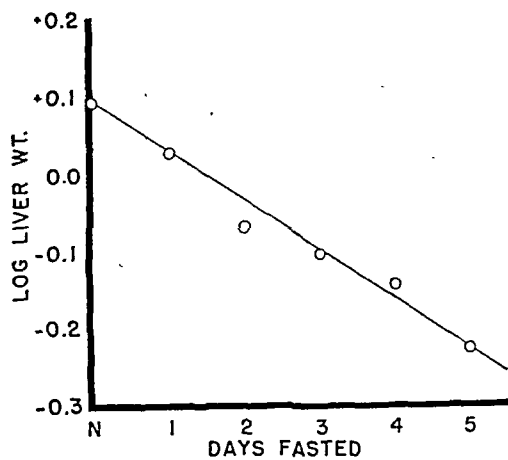


FIG. 1.

Logarithm (base 10) of moist liver weight in g plotted against the days fasted. The linear tendency is evident.

by the fifth day. The calculated equation for the average percentage body weight loss based on nearly 300 fasting mice is as follows: % weight loss = $1.10 + 7.48 \times \text{days fasted}$.

Liver Weight Loss. A logarithmic decrease in liver weight was found (Fig. 1); the liver weight dropped from about 5.5% of the body

weight in the normal mouse to 2.9% of the initial body weight in the mouse fasted 5 days. This corresponds to a loss of about 52% of the initial liver weight. The fact that the liver loses weight more rapidly than the carcass has been repeatedly observed (Table II).

Liver Total Lipid. The milligrams of total lipid for whole liver increased 2- or 3-fold during the first 2 fasting days (Table III); thereafter, it dropped to an amount significantly less than normally present. Expressed as percentage of moist liver weight the total lipid values are as follows: normal—7%, fasted one day—12%, two days—17%, three days—7%, four and five days—6%. One mouse after one day's fast had 17% fat in its liver; 2 mice on the second fasting day had 28 and 29%, respectively, of liver fat. The increase in liver total lipid has been shown to be due to an increase in the neutral fat fraction and to occur despite a decrease in the phospholipid content. It can be reasonably assumed that the fat which appeared in the fasting liver had its origin in a mobilization of depot fat.

Summary. Young adult male albino mice

TABLE II.
Data on Body and Liver Weights of the Normal and Fasting Mice.

	No. mice	Body weight					
		Initial		Carcass		Liver weight	
		Mean (g)	S.D.	Mean (g)	S.D.	Mean (g)	S.D.
Normal	15	20	1.3	18.7	1.5	1.099	0.16
Fasted 1 day	18	21	1.2	—	—	0.920	0.08
" 2 "	15	21	0.8	—	—	0.836	0.21
" 3 "	15	20	0.8	14.6	1.3	0.865	0.20
" 4 "	22	22	0.9	15.6	1.3	0.696	0.10
" 5 "	11	20	1.0	12.2	1.0	0.578	0.08

TABLE III.
Combined Summary Data on Liver Moist Weight and Total Lipid of Normal and Fasting Mice.

Condition	Liver moist weight*			Liver total lipid†		
	No.	Avg, g	S.D.	No.	Avg, mg	S.D.
Normal	55	1.24	0.26	34	70	17
Fasted 1 day	58	1.06	0.15	37	108	30
" 2 "	55	0.85	0.18	35	123	56
" 3 "	55	0.79	0.17	34	51	24
" 4 "	62	0.71	0.13	39	32	16
" 5 "	11	0.58	0.08	10	38	5

* Data from references 1 and 2 plus current data.

† Data from reference 2 plus current data.

lose about one-third of the initial body weight during a 5 day fast. The liver weight decreases logarithmically. In the first 2 days of fasting a marked increase was observed

in total liver lipid of young, male mice. Thereafter a decrease to less than normal values followed on the third to fifth fasting days.

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Studies on Q Fever: Complement-Fixing Antibodies in Meat Packers at Fort Worth, Texas.*

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Previous studies^{1,2} have suggested that risk of infection with Q fever is greater among stockyard and packinghouse workers than among the general population. The recovery of *Rickettsia burneti* from ticks in Liberty County, Texas,³ and the more recent demonstration of the same organism as the causative agent of a natural outbreak of Q fever among stock handlers and slaughterhouse workers at Amarillo, Texas,⁴ stimulated interest in the possibility of unrecognized human infections in this area. Accordingly, a serologic survey was undertaken to determine the frequency of complement-fixing antibodies to *R. burneti* in a group of packing plant employees at Fort Worth, Texas.

* These studies are being aided by grants from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., and the Rose Lampert Graff Foundation, Los Angeles, Calif.

¹ a. Derrick, E. H., *Med. J. Australia*, 1937, **2**, 281; b. Freeman, M., Derrick, E. H., Brown, H. E., Smith, D. J. W., and Johnson, D. W., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 193; c. Derrick, E. H., *J. Hyg.*, 1944, **43**, 357.

² Shepard, C. C., *Am. J. Hyg.*, 1947, **46**, 185.

³ Parker, R. R., and Kohls, G. M., *Pub. Health Rep.*, 1943, **58**, 1510.

⁴ a. Topping, N. H., Shepard, C. C., and Irons, J. V., *J. A. M. A.*, 1947, **133**, 815; b. Irons, J. V., and Hooper, J. M., *J. A. M. A.*, 1947, **133**, 815; c. Irons, J. V., Murphy, J. N., and Wolfe, D. M., *J. A. M. A.*, 1947, **133**, 819; d. Cox, H. R., Tesar, W. C., and Irons, J. V., *J. A. M. A.*, 1947, **133**, 820.

Serum specimens were obtained from the laboratory of the Fort Worth Health Department. The blood samples were collected during May and June, 1947, for the performance of routine serological tests for syphilis, from employees of meat packing plants. The sera used in this study were obtained only from workers who handled meat or meat products in raw or prepared states. Of the 1,433 specimens examined, approximately $\frac{3}{4}$ were secured from employees of one large packing house, the remainder being obtained from workers at a number of smaller establishments. When the routine tests for syphilis were completed, the sera were transported to this laboratory and stored in the deep freeze cabinet until tested for antibodies against *R. burneti*.

The Q fever antigen† was prepared from yolk sacs of embryonated eggs infected with a strain of *R. burneti* (American Nine Mile) isolated in Montana in 1935.⁵ The antigen consisted of a washed rickettsial suspension prepared by a combination of ether extractions to remove the fats and repeated centrifugation cycles to free the rickettsial bodies from extraneous proteins and fats. All tests

† The authors are indebted to Dr. Herald R. Cox, Virus and Rickettsial Section, Lederle Laboratories Division, American Cyanamid Company, for generously supplying the yolk sac antigen (lot No. Q 9M-5-16) used in these studies.

⁵ Davis, G. E., and Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2259.

were performed with a single lot of antigen. Complement-fixation tests were carried out according to the method described by Bengtson.⁶ The antigen was diluted 1:32 (2 units). Complement was titrated in the presence of antigen; 2 units of complement were used in the tests.

Serial 2-fold dilutions of inactivated sera were made, using a single pipette for each serum specimen. The hemolytic system consisted of equal volumes of 2% sheep erythrocytes and amboceptor made up to 2 units. In performing the tests, 0.2 cc of diluted antigen, serum, and complement were added to each tube, mixed thoroughly, and stored at ice-box temperature for 18 hours. Sensitized sheep erythrocytes (0.4 cc) were then added, and the tubes held at 37°C in a water bath for one hour or until control tubes showed complete hemolysis. Appropriate serum and antigen controls, as well as known positive and negative human and guinea pig sera, were included in each series of tests. The end point was read as the highest serum dilution showing at least 3+ fixation.

All sera were examined in a "screen" test using 2 dilutions of serum: 1:8 and 1:16. Sera having titers of 1:16 in the "screen" test were retested in 2-fold serial dilutions from 1:8 to 1:512 or greater.

Complement-fixing antibody titers against the *R. burneti* antigen are recorded in the accompanying table. Of the 1,433 specimens examined, 81 were anti-complementary and

1,238 showed less than 3+ fixation in a serum dilution of 1:8. One hundred and fourteen of the sera (8.0%) showed titers of 8 or greater, while 80 (5.6%) had a titer of 16 or more and 32 (2.3%), 32 or more. Seventeen serum specimens (1.2%) showed titers of 64 or more; among these, 10 had a complement-fixing antibody titer of 64, 2 a titer of 128, 3 a titer of 256, and 2 a titer of 512.

The individuals with antibodies for Q fever worked in various departments of the plants and there was no clear evidence that the positive reactors were engaged in any particular job. Some suggestive evidence was found that the higher titers occurred in individuals dealing with cattle rather than in those engaged in work with hogs and sheep. Epidemiological significance cannot be attached to these findings since the total number of individuals employed or tested in each department at the time of the study is unknown.

Among all the specimens examined, serological tests for syphilis† (Kahn, Kolmer) were positive in 39 instances (2.7%) and doubtful in 19 (1.3%). Among the 114 sera showing complement-fixing antibody titers of 8 or more against *R. burneti*, 4 (3.5%) had positive serological tests for syphilis and 2 (1.8%) had doubtful tests. The complement-fixation titers for *R. burneti* among these 6 individuals with positive or doubtful serological tests for syphilis were 8 in 2 instances, 16 in 3, and 512 in 1. The findings suggest only a coincidental relationship between serum reactivity with antigens for syphilis and Q fever.

Discussion. Considerable evidence exists to indicate that the complement-fixation test with yolk-sac antigens of *R. burneti* is reliable and specific for the serologic diagnosis of Q fever. In proved infections in which *R. burneti* was recovered from the blood stream, a rise in titer of complement-fixing antibodies has been demonstrated during convalescence.^{4,7} Serological cross-reactions with other rickettsial diseases do not occur;^{4,6,7} but it should be noted that there is

TABLE I.
Complement-Fixation Titers Against *R. burneti* in
Sera from Meat Packers at Fort Worth, Texas.

Titer*	No. of serum specimens	%	Total % positive
AC	81	5.7	
<8	1238	85.7	
8	34	2.4	
16	48	3.3	
32	15	1.1	
64 or >	17	1.2	8.0
Total	1433	99.5	

* Highest serum dilution yielding 3+ or greater fixation.

AC Serum anticomplementary.

⁶ Bengtson, I. A., PROC. SOC. EXP. BIOL. AND MED., 1941, 46, 665.

† These tests were performed by Mrs. Edith Mazurek in the laboratory of the Fort Worth Health Department.

little available information with regard to possible cross-reactions with other non-rickettsial diseases, with the exception of primary atypical pneumonia.^{7,8} However, agglutination tests with *R. burneti* have been negative in a variety of other diseases.⁹

It is not yet certain whether non-specific complement-fixation may occur with egg yolk sac antigens of *R. burneti*. In this laboratory a number of sera which reacted with the egg yolk sac antigen of *R. burneti* were also tested with an antigen prepared from uninoculated normal egg yolk sacs, but in no instance was complement fixation accomplished. Non-specific complement-fixation with syphilitic sera has been reported with a variety of other egg yolk sac antigens.^{10,11} Further studies with regard to these problems are in progress in this laboratory.

The significance that can be attached to the results of a complement-fixation test for Q fever on a single serum sample is uncertain, particularly when the serum titer is low (8 or 16) and when a clinical history of illness is unavailable. Early in convalescence considerably higher titers are to be expected^{2,4,7} but at longer intervals after infection lower titers might be anticipated. Elsewhere, however, it has been demonstrated that elevated serum antibody titers may persist for 1½ years after natural infection with Q fever.¹² Illness due to Q fever may vary in severity

from a trivial attack of "grippe"¹³ to fatal pneumonia,⁴ and subclinical infection may result in elevated antibody titers.^{2,4} It is apparent, therefore, that a history of illness may have little value in the interpretation of antibody titers obtained by serological survey. In the present study, the plant physicians were unaware of any unusual prevalence of undiagnosed febrile illness.

The results of the studies herein reported are believed to indicate the existence of unrecognized exposure to *R. burneti*. Similar data have not been reported in this country from other geographic areas or in other occupational groups. At present no conclusion can be drawn as to whether this frequency of occurrence of antibodies for Q fever is unusual. The occupation of these individuals may be immaterial and it is possible that a similar prevalence of antibodies for Q fever may be found in other occupational groups or in the general population in this area or elsewhere. Studies designed to elucidate some of these problems are in progress. Recent studies have indicated the occurrence of Q fever among persons exposed to dairy cattle in the Los Angeles area. *R. burneti* has also been isolated from the milk of such cattle.¹⁴

Summary. Complement-fixation tests employing an egg-yolk sac antigen of *R. burneti* (American Nine Mile strain) were performed on sera from 1,433 packinghouse workers at Fort Worth, Texas. Evidences of previous infection with *R. burneti* were obtained. Antibody titers of 8 or more were found in 8.0%, 16 or more in 5.6%, 32 or more in 2.2%, and 64 or more in 1.2%.

The authors are indebted to Dr. Harold M. Williams and Mrs. Edith Mazurek of the Fort Worth Health Department for their kind cooperation. The complement fixation tests were performed by Elizabeth L. Watson.

⁷ a. Robbins, F. C., and Ragan, C. A., *Am. J. Hyg.*, 1946, 44, 6; b. Robbins, F. C., Gould, R. L., and Warner, F. B., *Am. J. Hyg.*, 1946, 44, 23; c. Robbins, F. C., Rustigian, R., Snyder, M. J., and Smadel, J. E., *Am. J. Hyg.*, 1946, 44, 51; d. Robbins, F. C., and Rustigian, R., *Am. J. Hyg.*, 1946, 44, 64.

⁸ Dyer, R. E., *Am. J. Hyg.*, 1944, 30, 308.

⁹ Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1944, 44, 110.

¹⁰ Wertman, K., *J. Lab. and Clin. Med.*, 1945, 30, 112.

¹¹ Van der Scheer, J., Bohnel, E., and Cox, H. R., *J. Immunol.*, 1947, 56, 365.

¹² Sulkin, S. E., and Strauss, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 142.

¹³ Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, 44, 123.

¹⁴ Huebner, R. J., et al., *Public Health Rep.*, 1948, 63, 214.

Studies On Q Fever: Persistence of Complement-Fixing Antibodies After Naturally Acquired Infection.*

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Most of the information available with regard to the persistence of complement-fixing, agglutinating, or neutralizing antibodies in Q fever has come from the study of infections acquired accidentally by laboratory workers. Bengtson¹ demonstrated the persistence of complement-fixing antibodies in the sera of 2 laboratory workers 10 months after infection with Q fever. Huebner² reported that 14 individuals infected with Q fever in 1940 at the laboratory of the National Institute of Health escaped infection in the 1945-1946 outbreak. However, some of these individuals presumably were exposed to the agent in the laboratory at various times between the 1940 and 1946 outbreaks, and in addition, some of them may have been immunized with a vaccine which, it is believed, had some protective effect against *R. burneti*. In the Q fever outbreak in the Respiratory Diseases Commission laboratory,³ rickettsial agglutinin titers tended to decline after 2 months but in some cases were detectable 3 to 4 months after infection.

Little information is available concerning persistence of antibodies following Q fever acquired under natural conditions. Australian workers⁴ demonstrated rickettsial agglutinins in the sera of 2 individuals 17 months after naturally acquired Q fever; complement-fixation tests were not performed.

An outbreak of Q fever acquired under natural conditions at Amarillo, Texas, in

March, 1946,⁵ presented an opportunity to obtain data on the persistence of complement-fixing antibodies against *R. burneti*. It was felt that such data would be of value in the interpretation of the results of serological surveys for Q fever.⁵

The technique of the complement-fixation test, employing as antigen† yolk sacs of embryonated eggs infected with *R. burneti* (American Nine Mile), is described elsewhere.⁵ The end point of the test was read as the highest serum dilution showing at least 3+ fixation.

Post-convalescent blood specimens were obtained from a group of 17 persons who were ill with Q fever in March, 1946. The diagnoses were established on the basis of clinical illness compatible with Q fever, isolation of *R. burneti* from the blood of several patients, and a rise in titer during convalescence of complement-fixing or neutralizing antibodies for *R. burneti*.⁶ The blood samples obtained 5 to 7 weeks after onset of illness were tested in the laboratories of Dr. Herald R. Cox of Lederle Laboratories Divi-

* Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, **44**, 123.

⁴ Freeman, M., Derrick, E. H., Brown, H. E., Smith, D. J. W., and Johnson, D. W., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 193.

⁵ Strauss, E., and Sulkin, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 139.

⁶ a. Topping, N. H., Shepard, C. C., and Irons, J. V., *J. A. M. A.*, 1947, **133**, 813; b. Irons, J. V., and Hooper, John M., *J. A. M. A.*, 1947, **133**, 815; c. Irons, J. V., Murphy, J. N., and Wolfe, D. M., *J. A. M. A.*, 1947, **133**, 819; d. Cox, H. R., Tesar, W. C., and Irons, J. V., *J. A. M. A.*, 1947, **133**, 820.

† The authors are indebted to Dr. Herald R. Cox, Virus and Rickettsial Section, Lederle Laboratories Division, American Cyanamid Company, for generously supplying the yolk sac antigen (lot No. Q 9M-5-16) used in these studies.

* These studies are being aided by grants from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., and the Rose Lampert Graff Foundation, Los Angeles, California.

¹ Bengtson, I. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 665.

² Huebner, R. J., *Am. J. Pub. Health*, 1947, **37**, 431.

TABLE I.
Persistence of Complement-Fixing Antibodies
Against *R. burneti* Antigen in Subjects Ill with
Naturally-Acquired Q Fever.

Subject	Titer at indicated time after illness		
	5-7 wks	6 mo.*	17 mo.
1	1024	>32	64
2	256	>32	64
3	128	16	32
4	64	—	16
5	+	—	128
6	+	—	16
7	+	—	1024
8	64	32	—
9	256	32	—
10	256	>32	—
11	256	>32	—
12	128	>32	—
13	512	< 8†	—
14	128	>32	—
15	+	>32	—
16	+	32	—
17	+	8	—

Titer recorded as highest serum dilution yielding 3+ or greater fixation.

* Not tested in dilution greater than 1:32.

† Positive C.F. test; titer unknown.

‡ 8 was lowest serum dilution measured.

— Not tested.

sion, American Cyanamid Company, and Dr. J. V. Irons, Texas State Health Department. Elevated titers of complement-fixing antibodies were present for 17 months after infection, as shown in the accompanying table. In all but 3 subjects tested 6 months after illness, titers of 32 or greater were obtained; in only one subject did the titer fall below 8. Likewise, 17 months after infection, titers of 32 or more were obtained in the serum of 5 of the 7 individuals tested.

All of the complement-fixation tests were performed with antigens of the same strain of *R. burneti* prepared from the infected yolk sacs of embryonated eggs. However, different lots of antigen were used for the tests on the early (5-7 weeks) convalescent specimens and the late or post-convalescent specimens (6 and 17 months). It is recognized that exact comparison cannot be made of titers obtained in different laboratories or even in the same laboratory at different times. Nevertheless, experience has indicated that the general trend of the titers has comparative significance.

Discussion. The demonstration that high

titers of complement-fixing antibodies may persist for 1½ years after naturally acquired Q fever is important in both clinical and epidemiological studies of this disease. The clinical diagnosis of Q fever is difficult, particularly in sporadic cases. Diagnosis depends largely on the isolation of the rickettsia or on the results of serological studies. Because antibodies may persist for long periods the results of serological studies made during convalescence from a presumed attack of Q fever may be misinterpreted. As with other serological tests, the demonstration of a rise in antibody titer in convalescent phase as compared with acute phase blood specimens is of greater diagnostic value than a high titer present in a single specimen obtained during convalescence.

Little is known of the natural mode of infection with Q fever in this country. It is, therefore, conceivable that the persistence of elevated titers of antibodies in the subjects tested may be the result of continued contact with the rickettsial agent, subsequent to the initial illness. The unusual high titer in case No. 7, referred to in the table, suggests some such mechanism.

To determine whether or not Q fever may be endemic in the Amarillo area, 175 blood specimens from presumably normal individuals were tested for complement-fixing antibodies. These blood samples were collected for routine serological tests for syphilis and were obtained from the Amarillo City Health Department laboratory. Approximately 8% of these specimens showed complement-fixing antibodies against *R. burneti*. Only 2 individuals, however, had high titers (1:64 or more) and it was subsequently determined that both of these individuals had had Q fever during the epidemic. Clinical histories of previous illness were not available from other subjects who exhibited positive complement-fixation in low titers, and it is not possible, therefore, to assess the significance of these reactions.

Summary. Complement-fixation tests using an antigen of *R. burneti* (American Nine Mile) were performed on sera from a group of persons known to have had Q fever in 1946 in Amarillo, Texas. High titers of com-

plement-fixing antibodies were demonstrated 17 months after illness.

The authors are indebted to Dr. John M. Hooper

and Mr. Jack Wyatt of the Amarillo City Health Department for their kind cooperation. The complement-fixation tests were performed by Elizabeth Lee Watson.

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Effect of Digitoxin On Creatinine and Histamine Output of the Isolated Heart.*

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(Introduced by E. M. K. Geiling.)

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In the course of some experiments on the isolated hearts of rabbits, cats and rats which were perfused with digitoxin to measure the amounts of fixation of the drug, some unexpected observations were made. In using the modified Baljet reaction^{1,2,3} to determine the digitoxin colorimetrically, it was found that the intensity of the color in the perfusate was increased over that produced by the digitoxin in the perfusion fluid. This suggested the possibility that creatinine, which also gives the Baljet reaction, might be present in the perfusate. This was found to be the case. It was also observed that the perfusate contained a substance which caused a drop in the blood pressure of an anesthetized cat and a contraction of the isolated guinea pig intestine suspended in atropinized Tyrode solution. This led to the supposition that the perfusate contained histamine. Various investigators have found that the contracting

heart muscle produces histamine;^{4,5,6} however, this observation could not be confirmed by other workers.⁷ Since the creatinine and histamine output of the heart muscle might be related to its physiological activity, it seemed worth while to perform experiments in which the release of these substances could be correlated with the action of cardiac glycosides.

Experimental. The effect of digitoxin in a final concentration of 1:40,000 was studied on isolated rat, cat, and rabbit heart preparations. The animals were sacrificed by a blow on the head, and the heart was immediately removed and washed in oxygenated Ringer-Locke solution. The hearts were perfused through the coronary arteries by means of a cannula inserted into the aorta. The temperature of the perfusion fluid was kept at 36-39°C. Samples of perfusate were collected at intervals before and after the introduction of digitoxin into the system. Digitoxin was dissolved in 95% alcohol. Five ml of this solution contained 25 mg which diluted to 1000 ml gives a final concentration of 1:40,000. Control measurements showed that this amount of alcohol did not affect the motility of the heart and did not influence the creatinine and histamine output. The volume of fluid perfused per unit of tissue was not affected by digitoxin. In the case

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[†] Hoffman La Roche Fellow.

[‡] Guggenheim Fellow.

¹ Bell, J. C., and Krantz, F. K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 213.

² Krantz, F. K., and Bell, J. C., *Fed. Proc.*, 1947, **6**, 346.

³ Elmquist, A., and Liljestrand, A., *Acta Physiol. Scandinav.*, 1946, **12**, 53.

⁴ Eisa, E. A., *J. Roy. Egyptian M. A.*, 1947, **30**, 189.

⁵ Mareu, I., *C. R. Soc. de Biol.*, 1939, **130**, 573.

⁶ Anrep, G. V., Barsoum, G. S., and Talaat, M., *J. Physiol.*, 1936, **80**, 431.

⁷ Code, C. E., Evans, C. L., and Gregory, R. A., *J. Physiol.*, 1938, **92**, 344.

TABLE I.
Creatinine Excretion of Isolated Hearts.

Animal		Samples (Creatinine gamma per 10 ml per g heart)					
		1	2	3	4*	5*	6*
Rabbit	Exper.	0.48	1.14	1.03	1.30	1.08	0.89
	Control	0.68	0.63	0.89	0.83	0.61	0.85
Cat	Exper.	0.76	0.52	0.43	0.73	0.82	0.74
	Control	1.23	0.92	0.76	0.47	0.84	0.62
Rat	Exper.	3.72	1.38	1.22	3.55	1.88	1.84
	Control	3.14	2.35	5.01	2.03	2.05	5.21

* After digitoxin (1:40,000) perfusion started.

of the rabbit and cat hearts, the time which elapsed between the introduction of digitoxin and complete arrest of the heart were noted. The more resistant rat hearts were allowed to beat for an hour with digitoxin perfusion before the experiment was discontinued. The hearts were weighed at the end of each experiment. Ten ml samples of perfusate were collected for creatinine and histamine analyses. The samples were kept at ice-box temperature and analyses were carried out afterwards.

The substance in the perfusate which gave the Baljet reaction was identified as creatinine by use of the NC bacterial enzyme suspension of Dubos and Miller.^{8,9} The Jaffe alkaline picrate reaction was applied to samples of the perfusate before and after incubation for 90 minutes at 37°C with micro-organisms capable of destroying creatine and creatinine under aerobic conditions. The true creatinine was calculated from the difference in the color reaction before and after incubation. Two 4 ml aliquots of each perfusate sample were taken; one was subjected directly to colorimetric reaction with 2 ml of alkaline picrate, whereas the other was incubated for 90 minutes with 1 ml of cell suspension and 0.25 ml of M phosphate buffer, pH 7.0. Since the amount of protein present was less than could be precipitated by tungstate, the preparation of a tungstate filtrate was omitted.

Care was taken to use a cell suspension of potency sufficient to metabolize all the creatinine present. After incubation, separation was carried out by centrifugation. To 4 ml of the supernatant liquid, 2 ml of alkaline picrate was added. The alkaline picrate solution was prepared from 1 volume of 10% NaOH in 5 volumes of freshly saturated picric acid. After the solution had been allowed to stand for 15 minutes, it was added to standard and unknown solutions in a ratio of 1 alkaline picrate to 2 of the other solution. The color was allowed to develop for 10 minutes and was then measured using a Coleman Universal Spectrophotometer, Model 11, with the PC-4 filter, and at a wave length of 525 mμ. A solution of 1 volume of alkaline picrate and 2 volumes of distilled water was used as the blank. The creatinine content of the perfusates was calculated from the readings on standard creatinine solutions and expressed in gamma per 10 ml of perfusate per gram of heart.

The histamine determinations were carried out as follows. Essentially the histamine extraction method of Barsoum and Gaddum¹⁰ was applied. Ten ml samples of perfusion fluid were acidified with 1 ml concentrated HCl. The acidified perfusate was boiled for 2 hours, then evaporated to dryness, and extracted with 5 ml 95% ethyl alcohol saturated with NaCl. The alcohol was evaporated, and the residue taken up in 2 ml of water. This solution was tested on the atro-

⁸ Miller, B. F., and Dubos, R., *J. Biol. Chem.*, 1937, 121, 429, 447, and 457.

⁹ Miller, B. F., Allinson, M. J. C., and Baker, Z., *J. Biol. Chem.*, 1939, 130, 383.

¹⁰ Barsoum, G. S., and Gaddum, G. H., *J. Physiol.*, 1935, 85, 1.

TABLE II.
Statistical Analysis of Creatinine Results.

No. of animals	No. of samples		Mean values		T values*
	Before	After	Before	After	
10 Rabbits	21	22	0.97	0.90	1.4
10 Cats	21	20	0.69	0.73	1.5
7 Rats	14	16	4.54	3.84	1.9

* Significance of differences between the means; less than 3.0 is not considered statistically significant.

$$T = \frac{M_1 - M_2}{\sqrt{E_1^2 + E_2^2}} \quad (E = \text{mean error, } M = \text{mean}).^{11}$$

TABLE III.
Histamine Excretion of Isolated Hearts.

Animal		Samples (Histamine, gamma per 10 ml per g heart)					
		1	2	3	4*	5*	6*
Rabbit	Exper.	.034	.036	.033	.015	.008	.000
	Control	.030	.026	.030	.028	.028	.030
Cat	Exper.	.019	.020	.017	.009	.007	.003
	Control	.014	.018	.010	.015	.013	.018
Rat	Exper.	.11	.11	.12	.11	.11	.11
	Control	.08	.07	.12	.11	.10	.10

* See Table I.

TABLE IV.
Statistical Analysis of Histamine Results.

No. of animals	No. of samples		Mean values		T values*
	Before	After	Before	After	
11 Rabbits	26	25	.033	.0095	6.71
10 Cats	16	18	.0135	.0053	7.52
10 Rats	22	23	.13	.13	0.00

* See Table II.

pinized guinea pig intestine in the usual way. The sensitivity of the intestine was frequently tested with known amounts of histamine. This extraction method proved to be quite useful for estimating histamine in perfusates containing digitoxin. The method eliminates digitoxin and potassium ions, which, if present in the original perfusate, cause contraction of the guinea pig ileum.

Results. The results are summarized in tables wherein typical experimental data and the statistical analyses of a large number of experiments are presented. The creatinine output in each of the 3 species investigated was fairly constant and not affected by digi-

toxin (Tables I and II). The highest creatinine output was observed in rat hearts. The creatinine outputs of rabbit and cat hearts were approximately the same.

On the other hand, the histamine output showed an interesting correlation with the action of digitoxin in rabbit and cat. In these two species there was a significant decrease in the histamine content of the perfusate, which in many cases reached zero in the last sample. However, the histamine output of the rat heart was not affected (Tables III and IV).

It is of interest to note that the time elapsing before complete arrest of auricles and ventricles of the isolated hearts varied with the species. The most resistant was

¹¹ Burn, J. H., *Biological Standardization*, 1937, Oxford University Press, London, p. 29.

TABLES V AND VI.
Comparison of the Sensitivity of Rabbit, Cat, and Rat Heart to Perfusion with Digitoxin,
1:40,000.

TABLE V.

	No. of exper. animal	Amt of digitoxin (1:40,000) ml/g heart to cause heart arrest	Survival time in min.
Rabbits	16	11.5	18.0
Avg heart wt equals 9.4 g	17	15.9	11.0
	18	13.2	12.5
	19	17.0	10.3
	20	20.2	11.0
	21	12.5	7.0
	23	—	11.0
	24	12.4	11.0
		M = 15.9*	M = 11.5*
Cats	1	11.4	10.5
Avg heart wt equals 14.9 g	2	21.7	16.0
	3	21.5	13.0
	4	11.3	7.0
	5	26.3	13.8
	8	29.6	10.0
	10	—	15.0
	11	23.6	13.0
	12	13.5	8.5
		M = 19.8*	M = 11.9*

* M = Mean values.

TABLE VI.

Rats—All hearts beating well at one hour after the perfusion started. Average heart weight equals 1.45 g.

No. of exper. animal	Amt of digitoxin perfused in one hour
1	42.0 ml g/hour
2	126.2 "
5	87.1 "
7	153.8 "
8	100.0 "
	M = 101.8* ml per gram per hour

* M = Mean values.

the rat heart, while the rabbit and cat showed essentially similar sensitivity (Tables V and VI).

Discussion. From these results, it is reasonable to assume that the toxic effect of digitoxin on the isolated heart results in decreased histamine output, while the appearance of creatinine in the perfusion fluid is not altered. It has been shown by Anrep and coworkers⁶ that when the heart fails the histamine output diminishes. Our results with digitoxin-poisoned hearts corroborate these observations. It is interesting that the relative sensitivities of the 3 species to digitoxin are parallel to their relative sensitivities to histamine. The biochemical mechanism of

these findings is being investigated in further detail in order to gain a better understanding of these phenomena.

Summary. The perfusion fluid from isolated hearts of cats, rabbits and rats contains creatinine and histamine. There is a species variation in the excretion of these substances. When digitoxin (1:40,000) is added to the perfusion fluid, the creatinine output of the heart is not significantly altered in the 3 species studied. However, the histamine output of the cat and the rabbit heart decreases, while that of the rat heart remains unchanged. The isolated rat heart is much more resistant toward digitoxin than the cat and rabbit hearts.

Experimental Leptospirosis Infection in Chickens.*

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While the number of mammals found to be leptospiral carriers is steadily mounting, nothing is known about such infections in domestic fowl or other birds. Cases of leptospirosis have been reported among personnel of poultry farms and among poultry dressers but rats have always been considered the source of infection, and the possibility that chickens might be infected from rats seems to have been neglected. It seemed of interest, therefore, to examine the susceptibility of chickens to experimental leptospiral infection.

Since no virulent strain of *Leptospira ictero haemorrhagiae* was available, a bovin strain of leptospira,¹ which causes severe infections in cattle and human beings, was used. A heavy growth of the organism on semisolid medium was diluted 1:3 in saline and 1 cc was inoculated subcutaneously into 32 chickens. The birds varied in age from 6 weeks to 5 months.

Twelve birds were bled from the heart daily for 10 days after inoculation and 0.2 to 0.5 cc of blood was inoculated into semisolid or Schueffner's medium or both. Cultures were incubated at 30°C and were examined weekly with the dark field microscope. Positive cultures were obtained from 2 birds, one on the fourth and one on the ninth day after injection. This low proportion of positive cultures may have been due to the fact that chicken serum when added to heavily inoculated Schueffner medium has a harmful effect on the organism and inhibits growth.

The bird with the positive blood culture on the 9th day showed no clinical signs of illness but serum taken at 3 weeks had an agglutinin titer of 1:800. When the animal was sacrificed at 4 weeks no pathological

changes were found and no organisms were seen in sections of organs stained by Levaditi's method. A calf which was injected with 1 cc of a culture of the isolated strain reacted with a short fever (41.5°C) and developed an agglutinin titer of 1:1600 against the bovine strain of leptospira.

The bird with the positive culture on the fourth day became sick on the 8th day and was sacrificed. Liver, spleen and kidney were cultured on semisolid medium and organisms were successfully cultivated from the liver where they were also demonstrated in considerable number in sections stained by Levaditi's method. The organisms were found within well preserved liver cells. In some areas the liver cells were detached from one another and numerous leucocytes were found in the capillaries. A few organisms were found in sections of the kidney but they could not be cultivated from this organ or the spleen. There were no gross pathological changes on autopsy.

Five chickens were found dead between the 9th and the 14th day after inoculation and 5 more were sacrificed between the 6th and 9th day. No specific changes were found in these birds on autopsy. No leptospires were demonstrable with certainty in Levaditi stained organ sections although argentophile coils of undetermined nature were seen a number of times.

All of the surviving birds were bled at one to 2 week intervals for serological tests. Sera in dilutions of 1:20 and 1:200 were tested for the agglutination of formalized suspensions of *L. icterohaemorrhagiae*† and the bovine leptospiral strain. Equal volumes of serum dilution and antigen were mixed in small test tubes and incubated at 37°C for 2 hours. The results were read the following

* This work was aided by a grant from the Palestine Board for Scientific and Industrial Research.

¹ Bernkopf, H., Olitzki, L., and Stuczynski, L. A., *J. Infect. Dis.*, 1947, **80**, 53.

† The Jackson strain kindly supplied by Dr. J. C. Broom, The Wellcome Laboratories of Tropical Medicine, London.

morning by examining uncovered drops with a low power objective with dark field illumination. All the pre-inoculation bleedings were negative and the antibody response was very variable. Of six, 6-week-old birds, 3 developed no agglutinins at all and 3 developed titers of only 1:40. Among the 2- to 5-month-old birds, levels of 1:800 to 1:8000 were reached and during a 2- to 3-month period of observation these titers did not fall appreciably.

Since the successful demonstration of infection in subcutaneously inoculated birds was demonstrated in only 2 instances the actual number of infections accomplished cannot be stated since the antibody response in older birds may have resulted from the antigenic stimulus of the inoculum. This possibility was eliminated in another experiment in which 5 birds were given the organism by feeding. Each bird received 1 cc of a fluid leptospiral culture drop by drop with a pipette, care being taken that the material was slowly swallowed and that the mucous membranes were not traumatized.

Three weeks later all 5 birds had developed specific serum antibodies in titers ranging from 1:800 to 1:3200. This established the susceptibility of domestic fowls to infection in a manner which could well occur in nature. The actual occurrence of infection in nature remains to be demonstrated.

More than 500 chicken sera, from several flocks, have been examined for their agglutinin titer with bovine leptospira and *L. icterohaemorrhagiae* and while titers of 1:20 and 1:40 were frequently observed these are not considered significant. Two sera were found with titers of 1:400 with both strains and one serum which was positive at 1:200 with the bovine strain only. These serological findings though not adequate in themselves, suggest the occurrence of leptospira infections in chickens in nature.

Summary. Domestic chickens were shown to be susceptible to experimental infection with a bovine strain of leptospira, when given subcutaneously or by mouth. The infection occurs in inapparent form, as a rule.

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Influence of Fluorine in Mottled Teeth on Dental Caries.*

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If compounds of fluorine are to be used as drugs in combating human dental caries,¹ eventually the manner of action must be explained in order to direct their most practical application. At present the literature assumes that it works (1) through a constant intake of fluorine-bearing water² or (2) by

direct chemical combination with the constituents of the tooth, introduced either prenatally³ or postnatally.⁴ The latter assumption has been contested by the presentation of data which shows that a significant effect of fluoride acquired after tooth eruption on induced rat caries is not evident.⁵ Using

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¹ Editorials, *J. A. D. A.*, 1947, 34, 345; *J. A. D. A.*, 1947, 34, 411.

² Dean, H. T., *Am. J. Ortho. and Oral Surg.*, 1947, 33, 49.

³ Cox, G. J., Matuschak, M. C., Dixon, S. F., Dodds, M. L., and Walker, W. E., *J. D. Res.*, 1939, 18, 481.

⁴ Armstrong, W. D., and Brekhuis, P. J., *J. D. Res.*, 1938, 17, 393.

⁵ McClure, F. J., *J. Nutrition*, 1941, 22, 391.

TABLE I.

Group	Condition of animal	No. of animals	No. of molar teeth examined	No. fractured cusps per animal	No. carious cuspal involvements per animal	No. carious teeth per animal
I	Normal	15	180	10.9	4.0	1.8
II	Extirpated salivary glands	12	144	4.9	36.6	10.6
III	Mottled molars	9	108	20.0	5.4	1.5
IV	Extirpated salivary glands and mottled molars	9	108	12.6	28.1	8.5

a technique for desalivating rats worked out in our laboratory,⁶ it has been possible to demonstrate that a continuous supply of fluorine will arrest caries in the absence of saliva.⁷ The present report is based upon further studies with desalivated animals. Using the technique in combination with a method for increasing the fluorine content of rat molars, also worked out in our laboratory,⁸ it has been possible to remove the influence of saliva in animals previously subjected to fluorine in sufficient amounts to cause mottling during prenatal life and test the influence of the fluorinized tooth acting alone against caries-producing conditions.

Experiments and Results. A total of 18 rats were obtained from three mothers which had been receiving daily concentrations of 300 to 350 parts per million of fluorine as potassium fluoride during pregnancy, and a total of 27 young were selected from five mothers which received no fluorine. All parents were from the same Wistar breeding stock. One-half of the fluorinized young and 12 of the 27 nonfluorinized young were subjected to total salivary gland extirpations at the age of 24 days. On the 42nd day all animals were placed on a caries-producing diet.⁹ The food and distilled water were given *ad lib*. Weights were taken every 4 days. After 104 days on the diet, the animals were chloroformed, the jaws were separated, and all molar teeth examined with a sharp explorer fine under a binocular microscope, magnification 30X. Several sagittal planes

of jaw segments were examined histologically in ground and decalcified section to confirm the accuracy of the diagnosis. Lesions were classified into simple fractures and carious cavities. Presumably mechanical fracture of rat molars precedes most experimental carious destruction. Male and female differences were not sufficiently significant to warrant separate consideration in any group.

Observation in Table I shows that the reduction of saliva greatly increases the incidence of rat caries. This also has been demonstrated in a previous publication by us.¹⁰ Normal animals on the caries-producing diet average 4.0 carious cuspal involvements and 1.8 carious teeth per animal; those with salivary glands extirpated average 36.6 carious cuspal involvements and 10.6 carious teeth per animal; those with mottled molars average 5.4 carious cuspal involvements and 1.5 carious teeth per animal; and desalivated rats with mottled molars average 28.1 carious cuspal involvements and 8.5 carious teeth per animal. The number of cavities in the lower teeth exceeded those in the upper for all animals examined. Because the average number of carious teeth in the third group is only slightly lower than normal it seems that the fluorine incorporated in the tooth substance has very little, if any, influence in retarding caries. The greater number of carious cuspal involvements in the same group gives added support to this assumption. The outstanding conclusions, however, are based upon a comparison of the last group with the remaining three. Desalivated animals with mottled molars show an increase of approximately 80% of carious

⁶ Cheyne, V. D., *J. D. Res.*, 1939, **18**, 457.

⁷ Cheyne, V. D., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 58.

⁸ Cheyne, V. D., *J. D. Res.*, 1942, **21**, 145.

⁹ Hoppert, C. A., Webber, P. A., and Canniff, T. L., *J. D. Res.*, 1932, **12**, 161.

¹⁰ Cheyne, V. D., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 587.

lesions over normal animals and only about 20% reduction compared to desalivated animals not subjected to fluorine in their prenatal period. The relatively low number of fractures in nonfluorinized animals subjected to desalivation suggests that early breaks rapidly become carious. The higher number of fractures in the normal animals indicates the reverse condition, but in the fluorinized animals of Groups 3 and 4 it would be difficult to say to what degree the high frequency of breaks was due to greater friability of hypoplastic fluorinized teeth.

Summary. Some of the factors operative

in suppressing rat caries have been tested in this experiment. By using animals in which the salivary glands have been removed it has been possible to test the relative effects of fluorine prenatally incorporated in the teeth. When the caries score of operated rats with mottled or fluorinized teeth are compared with that of nonfluorinized animals without glands there is a measurable reduction in caries. When the percentage of reduction is compared with the caries score of normal animals, however, there is evidence that the saliva offers greater protection than the fluorine in tooth substance.

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Prevention of the Anti-Curare Action of Epinephrine by Dibenamine.

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The effects of N, N-dibenzyl-beta-chloroethylamine (Dibenamine) on the responses of various visceral effectors to epinephrine have been studied by Nickerson and Goodman.¹ They have reported adrenolytic actions of Dibenamine in various visceral effectors which have an excitatory adrenergic innervation. Epinephrine is known to exert an anti-curare action at the skeletal neuromuscular junction.^{2,3} The mechanism of this action is unknown. It does not appear to be related to any influence on adrenergic neuro-effector systems. In the present study it is demonstrated that Dibenamine prevents the anti-curare action of epinephrine.

Methods. Seven dogs were used. Intravenous sodium pentobarbital was the anesthetic employed in 6 dogs. In one animal, intratracheal ether anesthesia was used following the administration of 15.0 mg morphine sulfate. The peripheral portion of the

cut peroneal nerve was drawn across 2 glass-shielded platinum electrodes, and single shocks of supramaximal strength were applied every 2 seconds by means of an electronic stimulator (Electrodyne, model 461). Relatively isometric contractions of the tibialis anticus muscle were recorded on a revolving drum by means of a Meyerhof type of myograph. The femoral artery of the same side was exposed for intra-arterial injections. The femoral vein of the opposite side was exposed for intravenous injection.

The curare preparation used was Intocostrin, 20 units per cc (E. R. Squibb and Sons). This preparation has the same potency as a solution containing 2.7 mg of anhydrous d-tubocurarine chloride per cc. The following general procedure was used in each of the 7 experiments. Intocostrin was diluted with isotonic saline so that each cc contained 2 units, and this was injected intravenously in amounts sufficient to depress the amplitude of muscular contraction to between one-fourth and one-half of the original height. Then epinephrine (Adrenalin, Parke-Davis and Co.) was injected rapidly intra-arterially. After demonstrating the anti-curare action of

¹ Nickerson, M., and Goodman, L., *J. Pharm. and Exp. Therap.*, 1947, 89, 167.

² Rosenblueth, A., Lindsley, D. B., and Morison, R. S., *Am. J. Physiol.*, 1936, 115, 53.

³ Wilson, A. T., and Wright, S., *Quart. J. Exp. Physiol.*, 1936-37, 26, 127.

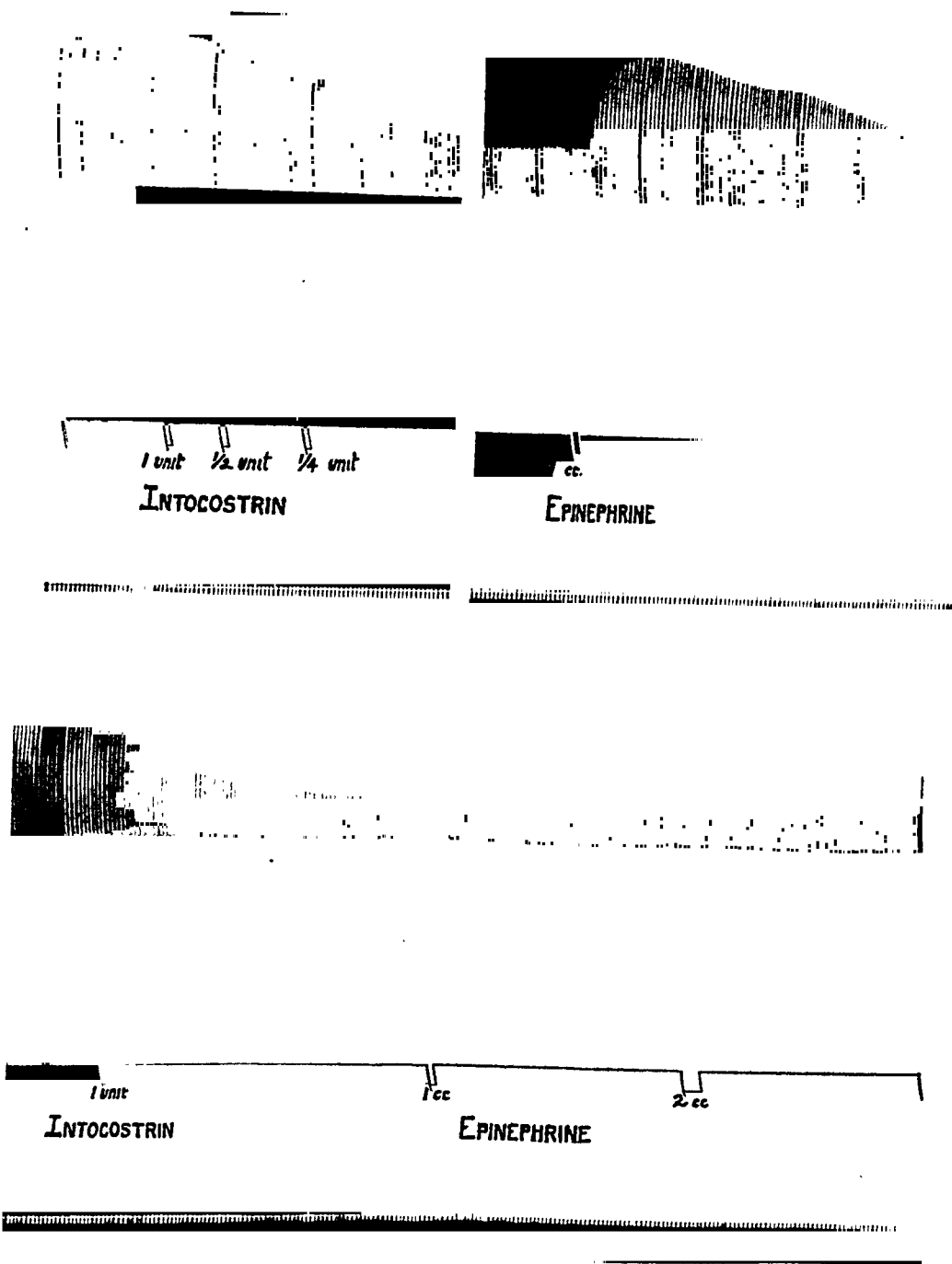


FIG. 1.

Prevention of the Anti-curare Action of Epinephrine by Dibenamine.

Upper Record. *Before Dibenamine.* From above downward record shows: (1) contractions of tibialis anticus muscle produced by stimulating the peroneal nerve at 2-second intervals. (2) times of intravenous injection of Intocostrin and intra-arterial injection of epinephrine

(1:100,000), and (3) time in 2-second intervals. At the break in the record a 3-minute section was removed, during which time an additional 0.25 unit of Intocostrin was injected.

Lower Record. After Dibenamine. Recording as in upper record. Animal had received 2 units of Intocostrin approximately 1 minute before the injection of Indocostrin indicated on the record.

epinephrine, Dibenamine was administered intravenously. The hydrochloride salt of Dibenamine was dissolved in propylene glycol (20 mg per cc) and then diluted with an equal amount of distilled water. Each dog was given between 25 and 45 mg of Dibenamine per kg within a period of 15 to 60 minutes. Within one to 3 hours after injection of Dibenamine, administration of curare and epinephrine was repeated.

Results. Intra-arterial injection of epinephrine (1.0 or 2.0 cc of a 1:100,000 solution of epinephrine in saline), following partial curarization, caused an immediate marked increase in the amplitude of muscular contraction in each of the 7 animals tested. A typical response is illustrated in Fig. 1, upper record.

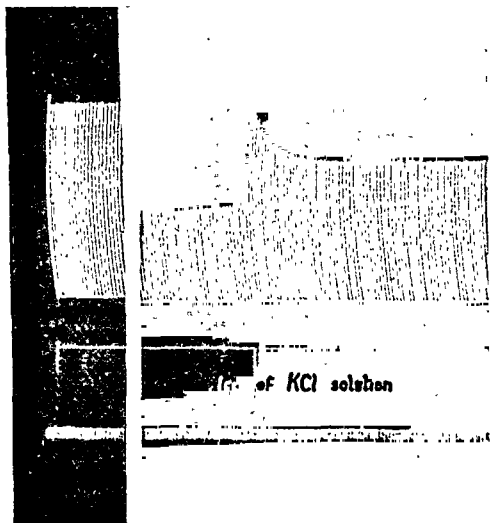


Fig. 2.

Anti-curare Action of Potassium after Dibenamine. Writing points are arranged as in Fig. 1. The record was obtained approximately 2 hours after administration of Dibenamine, 30 mg per kg. The break in the record indicates an 11-minute interval during which 1.2 units of Intocostrin were administered intravenously. Isotonic potassium chloride was administered intra-arterially.

After administration of Dibenamine, and subsequent partial curarization, the same dose of epinephrine failed to increase the amplitude of contraction in each of the 7 animals. An experiment is illustrated in Fig. 1, lower record. In fact, the injection of the epinephrine solution after Dibenamine and partial curarization produced a slight transient decrease in the amplitude of the contractions. The following controls indicate that the decreased amplitude was not due to the epinephrine but was related to the volume of the diluent.

1) In each of 2 dogs tested, injection of the same amount of epinephrine (20 μ g) in 0.02 cc of fluid (*i.e.* 0.02 cc of a 1:1,000 solution of epinephrine) failed to have any effect on the amplitude of contraction. 2) The same slight depression in amplitude of contraction was produced in 3 dogs by the intra-arterial injection of 1.0 to 2.0 cc of isotonic saline or water.

The effects of large doses of epinephrine were tested in three dogs. A dose of epinephrine 100 times that used to demonstrate anti-curare action before Dibenamine had no anti-curare effect after Dibenamine.

Isotonic potassium chloride solution (1.15%), 1.0 to 5.0 cc was administered intra-arterially to 3 dogs following partial curarization. The well-known immediate increase in amplitude of contraction was noted both before and after Dibenamine. The anti-curare action of potassium chloride following the administration of Dibenamine is illustrated in Fig. 2.

Summary. The effects of Dibenamine on the anti-curare actions of epinephrine and of KCl have been studied in 7 dogs anesthetized with sodium pentobarbital or with morphine and ether. Dibenamine, in doses of 25-45 mg per kg, prevents the anti-curare action of epinephrine but does not prevent the anti-curare action of potassium chloride.

Alloxan Diabetes in Mice.

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Alloxan diabetes has been produced in rabbits, rats, dogs, cats, monkeys, pigeons, and guinea pigs.¹⁻⁵ Although Dunn *et al.*,⁶ state that alloxan causes islet lesions in mice, no mention was found in the literature of the production of alloxan hyperglycemia in this species. In the course of studies on the effect of hyperglycemia on susceptibility to bacterial infection, it became desirable to find a consistent means of inducing this condition in a small laboratory animal such as the mouse.

Materials and Methods. Male Swiss albino mice of the same age and weight were selected for each experiment. A fresh aqueous solution of alloxan monohydrate (4 mg/ml for intravenous injection and 6 mg/ml for intraperitoneal and subcutaneous injections) was used. Blood sugars were determined by the method of Reinecke.⁷ Animals with blood sugar levels over 200 mg% were considered hyperglycemic, since blood sugar determinations on 20 control mice on food and water *ad libitum* were all under 150 mg%.

In view of the wide variation of dosage and route of administration necessary to produce alloxan diabetes in different species of animals,^{1-5,8} the following procedures were carried out.

* Technical assistance of James Samsell.

† Present address: Naval Unit, Camp Detrick, Frederick, Md.

¹ Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

² Dunn, J. S., McLetchie, N. G. B., *Lancet*, 1943, **244**, 384.

³ Ruben, J. A., and Yardumian, K., *Science*, 1946, **103**, 220.

⁴ Banerjee, S., *Lancet*, 1944, **227**, 658.

⁵ Goldner, M. G., and Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 31.

⁶ Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.*, 1943, **55**, 245.

‡ Coleman Junior electrophotometer.

⁷ Reinecke, R. M., *J. Biol. Chem.*, 1942, **143**, 351.

a. Intravenous tail injections of doses varying from 50 mg/kilo to 300 mg/kilo of alloxan were given to 65 mice maintained on Purina Mouse Chow and water *ad libitum* before and after injection. Blood sugars were determined 48 hours after inoculation. Intraperitoneal and subcutaneous injections of similar dosages of alloxan were given to additional groups of mice on the same diet.

b. Since it has been demonstrated⁸ that fasting prior to injection increases the susceptibility of rats to alloxan, 131 mice fasted for 8 to 24 hours, were given alloxan intraperitoneally and fed Purina Mouse Chow immediately thereafter.

c. Hard⁹ reported that some animals of a given species are resistant to repeated injections of alloxan. A number of mice previously unaffected by an initial dose of alloxan were therefore reinjected with similar dosages and later tested for hyperglycemia.

d. In order to determine the duration of alloxan hyperglycemia blood sugar determinations were made on groups of diabetic mice at various times after the injection of alloxan.

e. Inasmuch as riboflavin, epinephrine, glutathione, cysteine, and niacin have been reported to protect against alloxan^{10,11,12} an investigation of their protective action in mice was made.

Results. The optimal dose of alloxan for the production of hyperglycemia in mice was found to be 100 mg/kilo injected directly into a tail vein. All of 50 mice given alloxan in this dosage were found to be hyperglycemic at the end of 48 hours, while none of the animals died (Table I) (A).

⁸ Kass, E. H., and Waisbren, B. A., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 303.

⁹ Hard, W. L., and Carr, C. J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 214.

¹⁰ Gajewski, J., personal communication, 1947.

¹¹ Lazarow, A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 441.

¹² Banerjee, S., *Science*, 1947, **106**, 128.

TABLE I.
The Mortality and Blood Sugar Levels of Mice 48 Hours After Inoculation of Alloxan.

No. of animals	Conditions prior to inj. of alloxan	Dose of alloxan mg/kilo	Route of injection	No. of deaths 48 hr	Blood sugar levels after 48 hr mg %			
					50-150	150-200	200-300	Greater than 300
(A)								
5	Food and water <i>ad lib.</i>	300	Intravenous	5	0	0	0	0
5		200	"	3				2
50		100	"	0	0	0	0	50
5		50	"	0	2			3
(B)								
18	Same	400	Intraper.	3	4	3	4	4
195		300	"	19	23	52	37	64
8		200	"	0	3	3	2	0
8		100	"	0	4	2	1	1
(C)								
6	Same	750	Subcut.	2	2	0	0	2
6		600	"	4	1	0	0	1
6		500	"	2	2	1	0	1
6		400	"	3	3	0	0	0
6		300	"	0	6	0	0	0
6		200	"	0	1	4	0	1
6		100	"	0	4	1	1	0
(D)								
10	Fasted 24 hr	400	Intraper.	3	0	0	1	6
12	" " "	300	"	0	0	0	0	12*
100	" 8 "	300	"	45	2	6	15	32
9	" 24 "	300	Subcut.	0	4	2	3	0

* Five of these mice died after 60 hours.

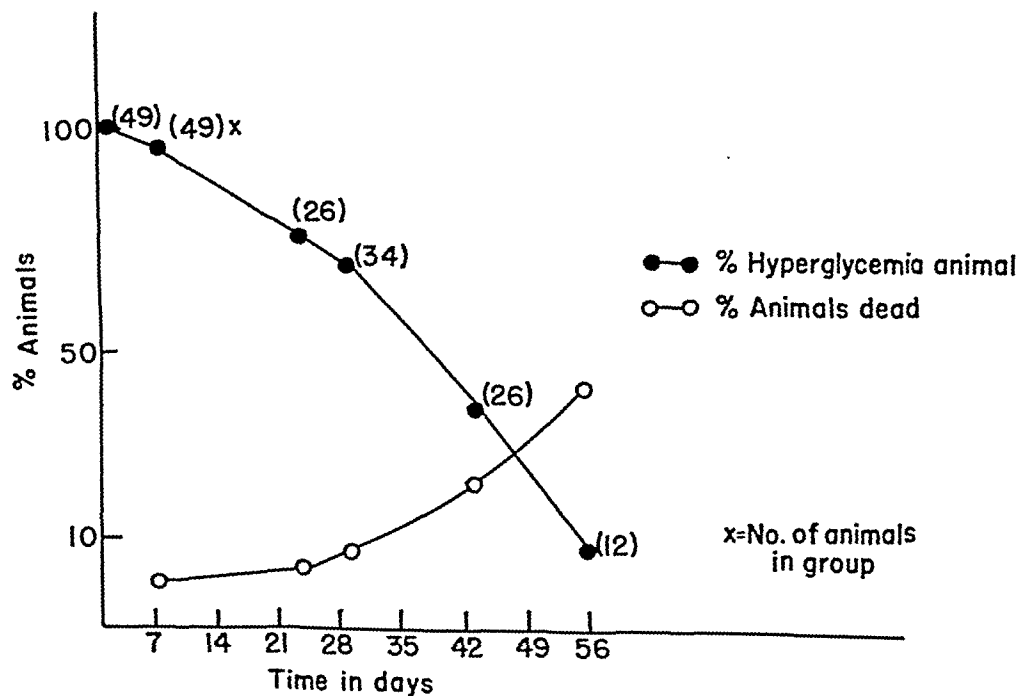


Figure 1.— Duration of hyperglycemia and mortality after inoculation with alloxan

TABLE II.
Effect of Riboflavin, Epinephrine, Cysteine, and Niacin on Development of Alloxan Hyperglycemia in Mice.

No. of animals	Compound tested	Route of inj.	Time given before inj. of alloxan*	Dose of compound	Death	Blood sugar levels after 48 hr, mg %				
						50-150	150-200	200-300	Over 300	Over 300
20	Riboflavin	Intraper.	6 hr	50 mg	16	1	0	2	1	1
20	"	"	6 "	10 "	3	5	7	1	4	4
20	"	Subcut.	6 "	10 "	0	1	3	1	15	15
60	Epinephrine	"	3 min.	.1 cc, 1:10,000	10	13	8	10	19	19
10	Glutathione	Intraper.	30 "	40 mg	8	2	0	0	0	0
20	Cysteine	"	1 hr	5 "	20†	0	0	0	0	0
20	"	Subcut.	30 min.	3 "	8	6	3	4	2	2
20	"	"	1 hr	1 "	4	0	4	3	9	9
20	Niacin	"	6 "	5 "	4	5	1	6	4	4
195	Control	—	—	—	19	23	52	37	64	64

* All mice were given 300 mg/kilo of alloxan intraperitoneally.

† All mice died before injection of alloxan.

The optimal dose for the intraperitoneal injection of alloxan was found to be 300 mg/kilo. Of 195 mice given this dose, 101 (52%) showed a hyperglycemia greater than 200 mg% in 48 hours (Table I) (B). Nineteen of the 195 (9%) mice died in 48 hours. Alloxan given in similar dosages by the subcutaneous route was found to be less effective in that only 6 of 42 mice so treated developed hyperglycemia (Table I) (C).

Fasting for 8 to 24 hours prior to injection increases the mortality due to alloxan without increasing the incidence of hyperglycemia in the survivors. Although only 60 of 131 (45%) fasted animals were hyperglycemic 48 hours after injection, 48 mice (37%) died in this interval (Table I) (D).

Five of 22 mice demonstrated to be resistant to an initial dose of alloxan were found to be hyperglycemic after a second injection of the same dose.

The diabetes produced by alloxan was found to be relatively transient in that at the end of 6 weeks 20 or 26 initially diabetic mice had survived and of these only 10 remained hyperglycemic (Fig. 1).

In the dosages used epinephrine, cysteine, niacin and riboflavin gave no significant protection against the action of alloxan (Table II).

Summary. The most effective dose and route of administration of alloxan were found to be 100 mg/kilo injected into the tail vein of the mouse. Fifty mice treated in this way all had blood sugar levels greater than 300 mg% 48 hours later. There were no mortalities.

Fasting before the intraperitoneal injection of alloxan increased the mortality in mice without increasing the incidence of diabetes among the survivors.

Mice resistant to the first injection of alloxan were not necessarily resistant to a second injection of the same dose.

Alloxan hyperglycemia was not permanent in all mice, since the blood sugar levels of one-half of the survivors reverted to normal within 6 weeks.

In the dosages administered epinephrine, cysteine, niacin and riboflavin did not give protection against the action of alloxan.

Tests of Possible Antagonism of Gold for Histamine Toxicity and Certain Allergic Reactions.*

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There has been considerable discussion about the possible allergic nature of rheumatoid arthritis as a disease process. The supporting evidence has been both clinical and experimental. According to Hench,¹ "no one has been able to set up any one particular syndrome as allergic arthritis or to prove that any of the arthritides are chiefly or solely the results of bacterial or food allergy." However, many attempts have been made to determine the possible role of bacterial allergy in the production of rheumatoid arthritis.² Klinge has reported the production of an arthritis in rabbits sensitized to horse serum by subsequently injecting horse serum into the joints.³ More recently Rich and Gregory have suggested that the characteristics of rheumatic carditis develop as a result of anaphylactic hypersensitivity.⁴

At the same time, increasing evidence has accumulated that gold salts favorably influence the clinical course of rheumatoid arthritis.^{5,6,7} In rats and mice the experimental arthritis produced by the pleuropneumonia-like organisms is prevented by the use of gold

salts and the disease once begun is arrested by their use.^{8,9}

In view of the favorable claims for an allergic factor in rheumatoid arthritis and because gold salts seem to alter favorably the course of the disease in some unknown way it was thought desirable to determine whether the gold ion could prevent histamine toxicity and anaphylactic shock and beneficially effect the Arthus phenomenon in animals. If it could be demonstrated that the gold ion altered beneficially experimental allergic reactions, the mode of beneficial action of gold in rheumatoid arthritis would be indicated, and conversely, if such experimental responses were not altered, this would suggest that gold acted in some other manner.

Anti-Anaphylactic Effect. Ten guinea pigs (weighing from 210 to 375 g) were each given 0.1 cc of horse serum hypodermically, and on the same day and every 3 days thereafter for 7 doses the guinea pigs were given 10 mg per kg of gold sodium thiosulfate intramuscularly. Twenty-eight days after the initial injection of the horse serum the animals were given 0.5 cc of horse serum intravenously and 11 of the 12 animals died in 5 to 15 minutes. Twelve control animals were given similar doses of horse serum without gold sodium thiosulfate, and only 1 animal survived in this group.

Acute Histamine Toxicity. Ten guinea pigs (weighing from 210 to 375 g) were given 10 mg per kg of gold sodium thiosulfate intramuscularly for 5 doses in a 7-day period and 4 of the animals died during the week. At the end of the week the 6 remaining animals, which had received gold were given 1 mg per kg of histamine phosphate intravenously, and they all died in 1 to 5

* This work was done under contract with the Office of Naval Research.

¹ Hench, P. S., Bauer, W., Ghrist, D., Hall, F., Holbrook, W. P., Key, J. A., and Slocumb, C. H., *Ann. Int. Med.*, 1938, **11**, 1089.

² Cecil, R. L., *Proc. Mayo Clinic*, 1940, **15**, 556.

³ Klinge, F., *Beitr. z. path. Anat., v. z. all. Path.*, 1929, **83**, 185.

⁴ Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, **72**, 65; *ibid.*, 1943, **73**, 239.

⁵ Freyberg, R. H., Block, Walter D., and Wells, G. S., *Clinics*, 1942, **1**, 537.

⁶ Ragan, Charles, and Tyson, T. L., *Am. J. Med.*, 1946, **1**, 252.

⁷ Freyberg, R. H., *Proc. Staff Meet. Mayo Clinic*, 1942, **17**, 534.

⁸ Sabin, A. B., and Warren, J., *J. Bact.*, 1940, **40**, 823.

⁹ Tripi, H. B., and Kuzell, W. C., *Stanford Med. Bull.*, 1947, **5**, 98.

minutes. Nine guinea pigs in the same weight range were taken as controls and given only the histamine phosphate in the same dosage, and they all died in 1 to 5 minutes after the injection.

Arthus Phenomenon. Rabbits were used to determine the possible ameliorating effects of gold sodium thiosulfate on the Arthus phenomenon. The method was as follows: 10 rabbits were sensitized by weekly subcutaneous injections of 1 cc of a 1 to 10 dilution of horse serum in physiological saline solution, according to the method previously used in this department.¹⁰ After sensitization was established, as shown by swelling at the site of injection, the rabbits were divided into 2 groups of 5 each. One group was given weekly intravenous injections of gold sodium thiosulfate beginning at 10 mg per kg and increasing to 30 mg per kg. The total dosage was 100 mg per kg in 6 weeks. The other group of 5 rabbits were kept as a control. The day following the intravenous injections of gold sodium thiosulfate, all 10 animals were given a subcutaneous injection of 1.0 cc of a 1 to 10 dilution of horse serum in physiological saline. The diameters of the resulting swellings were recorded at the end of 24 hours. The average diameters of the swellings in the control group ranged from 2.5 to 4.0 cm and in the gold treated group from 2.4 to 4.6 cm. Thus there was no demonstrable difference between the treated and untreated animals in the size of the

wheals resulting from the horse serum.

Gold was given intravenously to these rabbits as a matter of convenience and because this method is also used clinically.

Discussion. Evidently gold sodium thiosulfate given intramuscularly to guinea pigs offers no protection against anaphylactic shock, or the acute toxicity of histamine. Further, gold sodium thiosulfate does not affect the development of the characteristic wheal in the Arthus phenomenon; although, it should be stated that the antihistamine agents, pyribenzamine and benadryl, likewise fail to inhibit the development of this phenomenon.¹¹ However, Kyser, McCarter, and Stengle have recently reported that antihistamine drugs impede the development of myocardial lesions in rabbits injected with horse serum.¹² Since gold sodium thiosulfate does not appear to alter these manifestations of experimental sensitivity, it seems reasonable to assume that the beneficial action of gold therapeutically also does not rest on inhibition or suppression of allergic responses.

Conclusions. (1) Gold sodium thiosulfate failed to protect guinea pigs against lethal doses of histamine and anaphylactic shock, and did not demonstrably alter the magnitude of the Arthus phenomenon in rabbits. (2) These negative results suggest that the gold ion mediates its beneficial effects in rheumatoid arthritis in ways other than by amelioration of objective allergic manifestations.

¹¹ Dreisbach, R. H., *J. Allergy*, 1947, in press.

¹² Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. and Clin. Med.*, 1947, **32**, 379.

¹⁰ Chu, W. C., and Cutting, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 347.

Chemotherapy of Tuberculosis III. *In Vitro* and *In Vivo* Activities of Various Compounds.

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Continuing our studies of antituberculosis agents we have examined a heterogeneous group of compounds *in vitro*, and in addition, the sodium formaldehyde bisulfite addition product of 2-butoxy-5-aminopyridine has been evaluated *in vivo*. The results of these studies are set forth in the experimental part.

Experiments in vitro. The compounds, listed in Table I, were dissolved in Dubos' medium,¹ using the two-fold serial dilution method. The starting concentration was 64 mg%, or if the substance was not soluble to that extent, a saturated solution was used. Controls were included in every series, and each experiment was run in duplicate. The organisms were the pathogenic tubercle bacilli, H37RV and B1. The standard inoculum was 0.1 ml of a 14 day culture in Dubos' medium which had been diluted to an optical density of 0.2 as measured in the Lumetron colorimeter. Two weeks after inoculation tubes were examined for growth, and when readings were doubtful Ziehl-Neelsen stained smears were examined.

It is apparent from Table I, that none of the chemicals listed, with the exception of the p-aminosalicylic acid preparations, possess any great activity *in vitro* against tubercle bacilli. The differences in bacteriostatic activity between the different samples of p-aminosalicylic acid undoubtedly may be ascribed to differences in the purity of the samples. It should be noted that the findings with Compound 13 are completely different from those previously reported.² The differences in activities of Compound 13, and p-aminosalicylic acid from those previously reported^{2,3,4} are almost certainly due to important differences in technique. Compounds numbered 6, 7, 8, and 14 are being further studied. Compound 19, a commercial prod-

uct sold under the name "Mandelamine," has important antibacterial properties^{5,6} apart from its antimycobacterial activity. Its effect on experimental tuberculosis is now under study.

Experiments in vivo. Mice of the highly susceptible⁷ dba strain, average weight 20 g, were injected intravenously with 0.1 ml of a suspension containing 0.1 mg of *My. tuberculosis*, strain B1. One week after infection, the animals were divided into 3 groups. One group was given Compound 13 mixed into the ground diet in 2% concentration. A small group of mice was treated similarly, for comparative purposes, with p-aminosalicylic acid, the therapeutic effect of which is well known.^{3,4,8} The third group of mice remained untreated. The food cups were weighed daily and the amount of drug which each mouse ingested was estimated. The mice were weighed twice weekly. Surviving animals were sacrificed 32 days after infection. At autopsy the disease present in the lungs, liver, spleen, and kidneys was graded according to its extent and character. The scores for all organs were averaged and the average score, graded from 0 to 4+, was calculated for each group. A 4+ rating indicates caseous progressive tuberculosis of more than 50% of each organ.

The results of these experiments are given in Table II. While p-aminosalicylic acid

² Feinstone, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 153.

³ Lehmann, J., *Lancet*, 1946, **1**, 15.

⁴ Youmans, G. P., *Northwestern Univ. Bull. M. School*, 1946, **20**, 420.

⁵ Duca, C. J., and Scudi, J. V., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 123.

⁶ Scudi, J. V., and Duca, C. J., *J. Urol.*, in press.

⁷ Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, 1947, **86**, 159.

⁸ Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Proc. Staff Meets. Mayo Clin.*, 1947, **22**, 473.

¹ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE I.

Lowest Bacteriostatic Concentrations mg %* of the Various Compounds Which Inhibit the Growth of Strains of Pathogenic Mycobacteria. Dubos' medium, inoculum 0.1 ml, incubation time, 14 days.

Number	Compound	H37RV	B1
1	<i>p</i> -aminosalicylic acid HCl	0.125	0.125
2	" " " Lehmann†	1.0	1.0
3	" " " Youmans‡	1.0	1.0
4	<i>p</i> -phenetidin HCl	16.	32.
5	acetophenetidin	>32.	>32.
6	<i>p</i> -acetamidobutoxybenzene	8.	16.
7	diethylaminoethoxy-2,4,5-trichlorobenzene HCl	8.	—
8	" " -2-naphthalene HCl	8.	16.
9	3-hydroxy-4-methylpyridine	>64.	>64.
10	" " " -N-oxide	>64.	>64.
11	" " -4-methyl-1,2',3'-dihydroxypropyl-pyridinium chloride	>64.	>64.
12	2-methoxy-5-aminopyridine dihydrochloride	32.	>64.
13	2-butoxy-5- " sodium formaldehyde bisulfite	>64.	>64.
14	2-butoxy-5-acetamidopyridine	8.	32.
15	2',6'-diamino-2-butoxy-5,5'-azopyridine	>4.	>4.
16	2-amino-6-hydroxypyridyl-3,4'-azo-1'-hydroxybenzene	>4.	>4.
17	2-amino-6-hydroxypyridyl-3,4'-azo-1'-hydroxybenzene	>4.	>4.
18	<i>p,p'</i> -di(4-hydroxy-3-carboxy-phenylazo) diphenyl sulfone tetrasodium salt	—	32.
19	methenamine mandelate	16.	16.

* Numbers preceded by the symbol > indicate the concentrations of saturated solutions.

† Obtained through the kindness of Professor Jorgen Lehmann, Sahlgrenska Sjukhuset, Gothenburg, Sweden.

‡ Obtained through the kindness of Dr. Guy P. Youmans, Northwestern University Medical School, Chicago, Ill.

TABLE II.

Results of Treating Tuberculous dba Mice with *p*-Aminosalicylic Acid (No. 1), and with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13).

Compound No.	Drug conc. in diet	No. mice	Avg amt drug ingested g/kg/day	No. survivors	Amt tuberculosis
0	—	15	—	15	2+
13	2%	15	4.5	4	3+
1	2%	9	3.9	9	1+

exerts a favorable influence on tuberculous dba mice, Compound 13 appears to exert deleterious effects. Because the amount of Compound 13 ingested by the mice is more than twice its acute LD₅₀,⁹ it seems probable that the poor results may be in part attributed to toxic effects of the drug.

Guinea pigs were employed in the second *in vivo* test of Compound 13. Eighty-five tuberculin negative guinea pigs, average weight 300 g, were infected subcutaneously in the groin with 0.5 mg (moist weight) B1 per 500 g body weight. Eight days later,

when all animals reacted to 1 mg O. T. (Mantoux), they were divided into 5 groups and treated as follows:

Group 1. 20 guinea pigs received Compound 13 in their food in 2% concentration.

Group 1A. 15 guinea pigs, receiving the same treatment as Group 1, were used for blood studies.

Group 2. 20 guinea pigs received Compound 13 subcutaneously, 0.17 g per kg 3 times daily at 9:00 A. M., 5:00 P. M., and 12:00 M.

Group 2A. 15 guinea pigs, receiving the same treatment as Group 2, were used for blood studies.

Group 3. 15 guinea pigs were used as

⁹ Feinstein, W. H., Friedman, H. L., Rothlauf, M. V., Kelly, A. M., and Williams, R. D., *J. Pharm. and Exp. Therap.*, 1947, **80**, 153.

untreated controls.

All animals were weighed and tuberculin tests (Mantoux) with 1 mg O. T. were performed at weekly intervals. Blood, obtained by cardiac puncture from the animals in the subgroups, was used for the estimation of hemoglobin and Compound 13. Hemoglobin was measured in the Lumetron colorimeter and Compound 13 was measured according to the Marshall method.¹⁰ Blood for the latter test was drawn in the morning from the animals of Group 1A, and at specified intervals from animals of Group 2A. Each animal was autopsied as soon as possible after death. Surviving animals were sacrificed 90 days after infection. Upon autopsy the amount of tuberculosis involvement in the lungs, liver, spleen, and the lymphatic system was expressed in values proportional to the extent and severity of the disease. As in the preceding experiment, a rating of 4+ indicates that the organ exhibited widespread caseous lesions. The average ratings for the various organs represent a summary of the tuberculosis present in the animal. Representative blocks of tissue were sectioned and examined microscopically after staining with hematoxylin-eosin and with Ziehl-Neelsen stains.

Upon treating the tuberculous guinea pigs with the drug it was found that subcutaneous administration of the drug caused the appearance of broad shallow ulcers at the site of injection. These developed 2 to 3 days after treatment was started, were about 1 cm in diameter, and sometimes penetrated to the underlying muscle. Six or 7 such ulcers were present in most of the injected animals 2 weeks after beginning treatment. At autopsy, the muscle underlying these ulcers was found to be heavily congested over an area 3 to 4 times the size of the ulcer. At no time did any of the ulcers become visibly infected. After about a week the ulcers healed completely, leaving a scar covering the site of injection.

All the guinea pigs reacted to 0.1 mg O. T. (Mantoux) throughout the experiment. There were no significant differences between

the weight curves of the different groups of animals. Comparison of the hemoglobin 20 and 70 days after treatment was begun disclosed a 5 to 10% decrease. This decrease in hemoglobin is usually found in tuberculous animals after the disease has become widespread and progressive. No differences between the guinea pigs treated subcutaneously or orally were noted. Average concentrations of Compound 13 present in the blood are summarized in Table III. In terms of the previously reported *in vitro* activity² adequate concentrations of the drug were present at all times in the circulating blood, but as will be seen, these levels are completely ineffective in the treatment of experimental tuberculosis.

The amount of tuberculosis present at autopsy, and the percent of the animals which survived the full 90 days of the experiment, are listed in Table IV. Inspection of this table discloses no significant differences between the different groups either in the degree of tuberculosis or in the number of animals surviving. The same results were observed on histological examination of representative sections.

Discussion. The description by Dubos and Davis¹ of a liquid, synthetic medium in which pathogenic mycobacteria grow rapidly has provided investigators in the field with a most useful tool. In our hands, it has provided consistent and reproducible results. We, therefore, agree with the observation¹¹ that, "The use of Tween 80 medium has greatly facilitated the study of the effects of antibacterial agents upon mycobacteria." It was noted above that the findings with 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite are completely different from those previously reported,² and it was pointed out that the difference was due to important differences in technic. The results of the *in vivo* studies with this substance indicate that the drug is ineffective even in doses which produce serious toxic effects as evidenced by early death and severe local ulceration. These findings serve to emphasize the fact that the type of organism and the medium used will

¹⁰ Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

¹¹ Middlebrook, G., and Yegian, D., *Am. Rev. Tub.*, 1946, **54**, 553.

TABLE III.
2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13) Blood Concentrations in Guinea Pigs.*

Group	No.	Blood taken	Compound No. 13 mg % avg	Range mg %
1A	13	9 A.M.	.65	.33-1.13
2A	12	1 hr after injection	3.2	1.4-5.5
2A	9	3 " " "	1.0	.6-3.2
2A	11	5 " " "	.66	.44-1.1
2A	9	8 " " "	.23	.14-3.1

* Group 1A received Compound No. 13 in 2% concentration in the diet, and ingested it *ad libitum*.

Group 2A animals received the Compound subcutaneously, 0.17 g per kg 3 times daily at 9:00 A.M., 5:00 P.M., and 12:00 M.

TABLE IV.
Average Amount of Tuberculosis in Guinea Pigs Treated with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (Compound No. 13).*

Group	No. of guinea pigs	Lungs	Liver	Spleen	Lymph glands	Summary	% survivors
1	19	1.1	1.7	1.9	2.5	1.7	50
1A	14	1.7	1.7	2.1	2.6	2.3	34
2	18	1.8	1.5	1.9	2.9	1.9	30
2A	13	1.1	1.4	2.1	2.1	1.6	30
3	13	1.2	1.2	1.9	1.9	1.9	29

* Groups 1-1A received Compound No. 13 in 2% concentration in the diet.

Groups 2-2A received Compound No. 13 subcutaneously, 0.17 g/kg 3 times daily at 9 A.M., 5:00 P.M., and 12:00 P.M.

Group 3—controls.

The sub-groups (A) were bled by cardiac puncture for the blood studies listed in Table III.

profoundly influence the results. When 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite was tested *in vitro* using non-virulent mycobacteria growing in Proskauer-Beck medium,² the drug appeared to have a high order of activity. When tested against virulent tubercle bacilli growing in Dubos' medium the activity appeared greatly lessened. Since the compound is ineffective in the treatment of tuberculosis *in vivo*, it would appear that in this instance the latter method of testing gave a better indication of its *in vivo* activity.

The finding that Mandelamine (methenamine mandelate) possesses activity against virulent mycobacteria *in vitro* is of interest because this drug is known to be effective in

the treatment of urinary tract infections caused by non-acid-fast bacteria.¹² Its effect upon urinary tract tuberculosis is under investigation.

Conclusions. 1. The synthetic medium described by Dubos and Davis provides an efficient method of studying antimycobacterial agents. 2. Of 19 compounds tested *in vitro*, 4 showed sufficient activity to warrant further investigation. 3. 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite is ineffective in the treatment of experimental tuberculosis in mice and guinea pigs.

¹² Carroll, G., and Allen, H. N., *J. Urol.*, 1946, 55, 674.

Effect of Dibenamine on Blood Pressure in Normotensive and Hypertensive Subjects.

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Experimental evidence in animals showing that the sympathetic nervous system plays an important part in the mediation of reflex elevation of blood pressure has been presented by several authors.^{1,2,3} In hypertensive patients, lowering of the blood pressure has been induced temporarily by means of spinal⁴ or continuous caudal⁵ anesthesia, and for longer duration by extensive dorsolumbar sympathectomy.^{6,7,8} These facts are considered generally to support the theory that a neurogenic factor plays a primary role in the genesis of hypertension.

The clinical evaluation of this neurogenic factor has proven to be difficult. The available tests for determining the presence and degree of the neurogenic element are not satisfactory because they are non-specific or the drugs employed act too diffusely. Indeed, amylal is a central nervous system depressor, spinal and continuous caudal anesthesia block both autonomic and somatic functions, tetraethyl ammonium hydrochloride affects both sympathetic and parasympathetic ganglia⁹

and depresses the resting blood pressure of both normotensive and hypertensive subjects.^{10,11} The value of the results thus obtained is questionable.

A drug having a relatively prolonged effect and capable of blocking specifically sympathetic impulses would meet the requirements for a desired test. Dibenamine, a new sympatholytic agent, appears to possess these pharmacologic properties.

In order to investigate the role of the neurogenic factor in hypertension, a study of the effect of dibenamine on the blood pressure in normotensive and hypertensive subjects was undertaken.

Methods and Material. Dibenamine (N, N - dibenzyl - β - chloroethylamine - hydrochloride) is a new synthetic autonomic blocking agent. Structurally it is related to the nitrogen mustards. Functionally it is a highly specific sympatholytic and adrenolytic drug.^{12,13} The dibenamine* used in this investigation was supplied in ampules as a 5% sterile solution in 50% alcohol, acidified for stability. It was administered by the intravenous route. An infusion of a solution of 0.9% sodium chloride or of 5% glucose was first started. When this was flowing freely, the desired amount of dibenamine was added to the flask containing 300 to 500 cc of the saline or glucose solution and mixed thoroughly. The single dosage used averaged 5 mg per

¹ a. Heymans, C., and Bouckaert, J. J., *C. R. Soc. de Biol.*, 1931, **106**, 471; b. Heymans, C., *Surgery*, 1938, **4**, 487.

² Nowak, S. J. G., and Walker, I. J., *New England J. Med.*, 1939, **220**, 269.

³ Grimson, K. S., Kernodle, C. E., Jr., and Hill, H. C., *J. A. M. A.*, 1944, **126**, 218.

⁴ Gregory, R., and Levin, W. C., *J. Lab. and Clin. Med.*, 1945, **30**, 1037.

⁵ Russek, H. I., Southworth, J. L., and Zohman, B. L., *J. A. M. A.*, 1946, **130**, 927.

⁶ Craig, W. M., and Adson, A. W., *Surg. Clin. North America*, 1939, **10**, 969.

⁷ Peet, M. M., and Iseberg, E. M., *J. A. M. A.*, 1946, **130**, 467.

⁸ Smithwick, R. H., *Arch. Surg.*, 1944, **40**, 180.

⁹ Collier, F. A., Campbell, K. N., Berry, R. E. L., Suter, M. R., Lyons, R. H., and Moe, G. K., *Ann. Surg.*, 1947, **125**, 729.

¹⁰ Birchall, R., Taylor, R. D., Lowenstein, B. E., and Page, I. H., *Am. J. Med. Sc.*, 1947, **213**, 572.

¹¹ Haimovici, H., unpublished data.

* Dibenamine was supplied by the Giraudan-Delawanna, Inc., Delawanna, N. J., through the courtesy of Dr. W. Gump.

¹² Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

¹³ Haimovici, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 486.

kg of body weight. The total single dosage, regardless of weight, never exceeded 500 mg in our present study. The infusion was given in most cases over a total period of 60 to 75 minutes. At the completion of the dibenamine administration, the vein through which it was given was flushed with 100 to 150 cc of normal saline or glucose solution. (We found this procedure necessary for preventing local phlebothrombosis, which otherwise frequently followed dibenamine infusion in the early stage of this investigation).

The effects of this drug were studied 69 times in 38 subjects (18 normotensive and 20 hypertensive). Among the latter, 14 were suffering from essential hypertension and 6 from systolic hypertension associated with arteriosclerotic cardiovascular disease.

Arterial blood pressure was measured in the arm by the standard auscultatory method. The measurements were made in the supine, sitting and standing positions. The blood pressure (B. P.) and the pulse rate (P. R.) readings were taken at frequent intervals before, during and after the infusion. In most instances the observations were carried out over a period of 24 to 72 hours. All subjects were hospitalized patients, whose blood pressure was recorded over a period of a few days or weeks before the present tests were undertaken. In addition, the study of B. P. changes preceding and following dibenamine administration was done under standard, basal conditions. After the infusion the patients were kept in bed for about 12 to 24 hours.

Results. A. Effect of dibenamine on the blood pressure in the supine position.

1. Thirty tests were performed in 18 normotensive subjects (Table I). The average pre-injection B.P. was 124/73, the average post-injection B.P. was 122/69, with an average change of $-2/-4$. The range of variations in the post-injection B.P. was between $+30$ and -40 for the systolic and between $+20$ and -24 for the diastolic.

2. In hypertensive subjects the effect of dibenamine varied with the type of hypertension. The results obtained fall into 3 groups.

a. The first group (Table I) includes

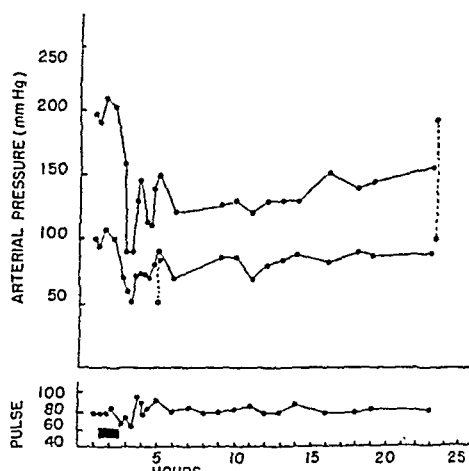


Fig. 1.

Typical effect of dibenamine (■) on the resting blood pressure and pulse rate in a case of benign essential hypertension. The vertical broken lines represent orthostatic blood pressure changes after one minute in the upright position.

those patients with essential hypertension whose B. P. fell to normal or below normal levels. Six out of the 7 subjects in this group had an uncomplicated essential hypertension (benign phase) and one had visceral changes characteristic of the malignant phase of the disease. In the 17 tests performed in this group, the average pre-injection B. P. was 180/112, the average post-injection B. P. was 132/86, with an average change of $-48/-26$.

The onset of the depression on the B. P. occurred occasionally during the infusion, but in most instances the fall began at or soon after the conclusion of the drug administration. The maximum fall was recorded usually within 30 to 60 minutes after the onset of the depression. However, in 2 instances a delayed response was seen, and the maximum depression of the B. P. was noted $2\frac{1}{2}$ and $4\frac{1}{2}$ hours respectively after the completion of the dibenamine infusion.

The return of the supine B. P. to the pre-injection level was gradual and took usually from 24 to 72 hours. Fig. 1 illustrates a typical response of a patient with moderately advanced essential hypertension.

The depressor effect of dibenamine was usually more marked after the second and subsequent injections than after the first one,

TABLE I.
Effect of Intravenous Injection of Dibenamine on the "Resting" Blood Pressure and Pulse Rate.

Group of patients	No. of subj.	No. of inj.	Pre-injection		Post-injection		Change in		
			B.P.	P.R.	B.P.	P.R.	Syst.	Diast.	P.R.
1. Normotensive	18	30	124/73 \pm 2.2/1.7*	78 \pm 2.0	122/69 \pm 3.0/1.9	84 \pm 2.8	- 2 \pm 2.6	- 4 \pm 1.5	+6 \pm 1.6
2. Essential hypertension (benign phase)	7	17	180/112 \pm 5.7/3.0	82 \pm 2.8	132/86 \pm 6.8/3.9	86 \pm 3.9	-48 \pm 3.6	-26 \pm 2.5	+4 \pm 2.1
3. Essential hypertension (malignant phase)	7	12	231/136 \pm 7.6/5.6	100 \pm 4.0	204/122 \pm 5.2/3.3	102 \pm 3.8	-27 \pm 4.3	-14 \pm 3.2	+2 \pm 1.1
4. Systolic hypertension (associated with A.S.C.V.D.)	6	10	177/81 \pm 3.8/3.6	82 \pm 6.6	170/79 \pm 5.9/2.7	83 \pm 4.5	- 7 \pm 4.8	- 2 \pm 2.0	+1 \pm 3.5

* Mean \pm Standard Deviation of the Mean.

† In this group, which responds to dibenamine, one patient presented visceral manifestations characteristic of the advanced phase of essential hypertension.

if an interval of only 2 to 4 days elapsed between the injections. This was noted even when the B. P. had returned to the pre-injection level during the interval, and might indicate a certain cumulative effect of the drug. Thus the average of the lowest B. P. after repeated dibenamine infusion was 107/75 as compared to 132/86, the average of the B. P. after all the injections.

b. The second group (Table I) includes 7 patients with essential hypertension whose B. P. did not change significantly after the administration of dibenamine. All the patients in this group had clinical manifestations of the malignant phase of essential hypertension (neuroretinitis, impairment of the renal function, E.C.G. changes indicative of myocardial damage, etc.). The effect of dibenamine on the B. P. in this group was either a small depression, no change or even a slight rise over the pre-injection values. When a fall in B. P. did occur, neither the systolic nor the diastolic pressures ever reached a normal level. The average pre-injection B. P. was 231/136, while the average post-injection B. P. was 204/122.

c. The third group of hypertensives includes 6 patients with systolic hypertension and arteriosclerotic cardiovascular disease (Table I). In this group the B. P. exhibited only slight variations, and the average pre-injection and post-injection values were practically the same (177/81 and 170/79).

B. Orthostatic changes. In most instances marked postural hypotension, accompanied by marked tachycardia (140 to 160), was seen following the intravenous administration of dibenamine, in both normotensive and hypertensive subjects. The orthostatic changes were present usually at or soon after the completion of the infusion and disappeared 12 to 24 hours thereafter. As a rule postural tachycardia outlasted by about 12 hours postural hypotension.

C. Other effects. The range of variations in the resting pulse rate was between +36 and -20, with an average change of +3. This applied to all groups of patients.

Measurement of skin temperatures of the head and the upper and lower extremities, under control conditions, were recorded in all

cases. Release of the sympathetic tone was apparent first in the head, second in the upper extremities, and last in the lower extremities. Delayed or absent responses were noted in the lower extremities of certain patients.

Observations on these effects and others (pupillary changes, sweat function, pain, etc.) will be reported elsewhere.

D. Side Effects. Side reactions due to the administration of dibenamine were either local or general. Local reactions included phlebothrombosis, which has been mentioned already under methods and materials, and pain in the arm along the vein during the infusion, which was seen in 4 instances. Most general reactions were minor. Dryness of the mouth and congestion of the nose were seen in practically all cases. Drowsiness and transient nausea were common. The major side effects included vomiting in 8 instances, mental confusion and emotional lability in 3 cases, and one case of mental confusion with loss of sphincteric control and deep sleep lasting one hour.

All side effects were transient. A slow, even rate of infusion and confinement to bed of the patient for about 12 hours after the injection, *i. e.* until the orthostatic hypotension could no longer be elicited, appeared important factors in the prevention or reduction of the side effects to a minimum.†

Comments. The administration of dibenamine resulted in a significant reduction of the resting B. P. of subjects with benign or moderately advanced essential hypertension only. These results are at variance with those presented by Hecht and Anderson.¹⁴ Orthostatic hypotension occurred in both normo-

tensive and hypertensive subjects.

There is ample evidence to show that dibenamine is a very potent and highly specific adrenolytic and sympatholytic agent.^{12,13} It probably acts directly on the neuro-effector cells, where it blocks the sympathetic impulses or the action of sympathomimetic substances (sympathin E, epinephrine). Thus the B. P. changes induced by dibenamine may be interpreted as being due to the blockade of sympathetic tone.

The lowering to normal levels of the resting blood pressure by dibenamine, in subjects with benign or moderately advanced essential hypertension only, tends to suggest that: 1. a neurogenic element, *i. e.* altered sympathetic tone is present in essential hypertension, and 2. the evaluation of this element by the administration of dibenamine may be of great help in the selection of patients when surgery is contemplated.

Because of its specific sympatholytic action, dibenamine offers a valuable tool for the investigation of the neurogenic component of a variety of vascular conditions. Further study is under way.

Summary. 1. Dibenamine hydrochloride, a new sympatholytic and adrenolytic drug was administered intravenously in the dosage of 5 mg/kg of body weight to normotensive and hypertensive subjects. 2. Reduction to normal or below normal levels in resting arterial blood pressure was observed only in benign or moderately advanced essential hypertension, but not in the malignant phase. Orthostatic hypotension occurred in both normotensive and hypertensive subjects. 3. The depressor effect started at the end of the infusion and lasted 24 to 72 hours. 4. All side reactions were transient, and major side reactions occurred only in a few instances. 5. The role of the neurogenic factor in essential hypertension is discussed. The usefulness of dibenamine in evaluating the neurogenic element is suggested.

† Much care was exercised by keeping the patient in bed during the phase of orthostatic hypotension because of the possible deleterious effect of the latter on the coronary circulation in advanced cardiovascular disease.

¹⁴ Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, **3**, 3.

Early Effects of Denervation Upon Response of Muscle to Continuous Stimulation.

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The gastrocnemius muscle of the normal rat can sustain a high rate of work for several days in response to direct faradic stimulation.¹ The purpose of this study was to determine the early effects of cutting the sciatic nerve upon the ability of the gastrocnemius muscle to sustain its responsiveness. It was found that the response of the denervated muscle to a standard stimulus was decreased below that of sham-operated animals within 24 hours after the nerve was cut and continued to decrease rapidly until responsiveness was almost completely lost.

Methods. Male rats of the Sprague-Dawley strain were used. The control and experimental animals were closely matched into pairs according to weight which ranged from 310 to 350 g. The sciatic nerve was cut just above the level of the knee joint. In the control animals the nerve was exposed but not cut. The operations in Exp. 1 were done under barbiturate anesthesia, and the animals were subjected to the work test immediately following operation. The operations in Exp. 2 and 3 were done under ether anesthesia, since there was a delay of 24 to 48 hours in starting the work test. The animals were prepared for the work test by the subcutaneous injection of phenobarbital sodium and the intraperitoneal injection of cyclopal sodium. Anesthesia was maintained throughout the experiment by the repeated subcutaneous injection of phenobarbital sodium.

The animals were placed upon an animal board, enclosed in a temperature cabinet (28°C) and prepared for the stimulation of muscle to lift 100 g 3 times per second, according to the procedure of Ingle.² A silver needle placed in the biceps of the thigh served as one electrode, and a hemostat, which was

clamped on the lower tibia, served as the second electrode. The muscular contractions were registered on automatic work adders. The relative amounts of work are expressed as recorded revolutions, and rates of work as number of recorded revolutions per minute (RPM). Each recorder revolution represents approximately 400 g-cm of work. All of the apparatus used was refined over that previously described² but the principles of method have remained the same. An electrical stimulator (Nerve Stimulator, Model B, Upjohn) was used which had adjustable amplitude, wave shape, pulse length and pulse frequency. A cathode ray oscilloscope was incorporated into the stimulator for observing and calibrating the output. A small amount of AC current was imposed on that of the DC pulse. The frequency of pulse was 3 per second, its duration was 20 milliseconds and its intensity was 12 milliamperes.

Experiments and Results. In Exp. 1, 12 rats having the sciatic cut and 12 rats having sham operations were subjected to direct stimulation of the muscle immediately following operation. The rates of work were recorded at the beginning of the experiment and 24, 30, 36, 48, 54, 60, and 72 hours later. The average rate of work for denervated muscle was depressed below that of the control animals at 24 hours and decreased rapidly thereafter so that only feeble responses were recorded after 48 hours. At the end of the 72-hour test period the intensity of the stimulus was increased from 12 to 20 milliamperes. The average rate of work increased from 0.5 to 13.2 RPM in the denervated muscles and from 10.2 to 15.7 RPM in the control series. The total number of recorder revolutions for the denervated series was 24,764 and for the control series 51,655.

¹ Heron, W. T., Hales, W. M., and Ingle, D. J., *Am. J. Physiol.*, 1934, **110**, 357.

² Ingle, D. J., *Endocrinology*, 1944, **34**, 191.

In Exp. 2, 12 rats were denervated under ether anesthesia, and 24 hours later they were anesthetized with the barbiturates and subjected to direct stimulation of the muscle. The initial level of work was subnormal and decreased rapidly so that muscular responsiveness was almost lost by 24 hours. When the intensity of stimulation was raised to 20 milliamperes the average rate of work increased from 0.1 to 18.5 RPM.

In Exp. 3, 12 rats were denervated and 12 rats were sham-operated under ether anesthesia, and 48 hours later they were anesthetized with the barbiturates and subjected to direct stimulation of the muscle for 24 hours. The initial rates of work were normal for the sham-operated rats and subnormal for the denervated series. Muscular responsiveness decreased rapidly in the latter series so that by 24 hours muscular contractions were very weak. When the intensity of stimulation was raised to 20 milliamperes the average rate of work was increased from 1.0 to 13.7 RPM in the denervated series and from 12.8 to 21.0 RPM in the control series. The total amount of work for the denervated

series was 6,638 recorder revolutions and 22,187 recorder revolutions in the control series.

The data on averages for rates of work per minute for all animals are summarized in Fig. 1.

Discussion. In these experiments the responsiveness of the gastrocnemius muscle was decreased below normal within 24 hours following the cutting of the sciatic nerve. The decrease occurred in those animals which were kept for 24 hours before stimulation was started as well as in the series which were stimulated continuously. The performance of work did hasten the "fatigue" of the denervated muscle, as is evident from the curves of average performance (Fig. 1) of those animals which worked continuously as compared with the performance of rats which were delayed for 24 and 48 hours following denervation.

The early effect of denervation upon the response of the muscle to direct faradic stimulation appears to be due to a rise in the threshold for adequate stimulation rather than an incapacity of the muscle to contract at all. When the intensity of the pulse was increased from 12 to 20 milliamperes at the end of the experiment the muscle contracted strongly again and sustained a high rate of work for several hours. The response was less than in the sham-operated control animals. In considering this effect it is important to recognize that our standard stimulus is optimal under ordinary experimental conditions. It will elicit an optimal amount of total work in normal animals during several days, although a temporary rise in rate of work can be induced by a more intense stimulus. A pulse of 20 milliamperes is sufficiently intense to cause tissue destruction within a period of a few hours.

The explanation of the early effect of denervation upon the ability of muscle to work is not known to us. The loss of muscular responsiveness may be due to metabolic changes caused by denervation possibly relating to the changes which result in muscular atrophy. An alternative explanation is that the dying of the nerve fibers removes an important excitatory mechanism from the

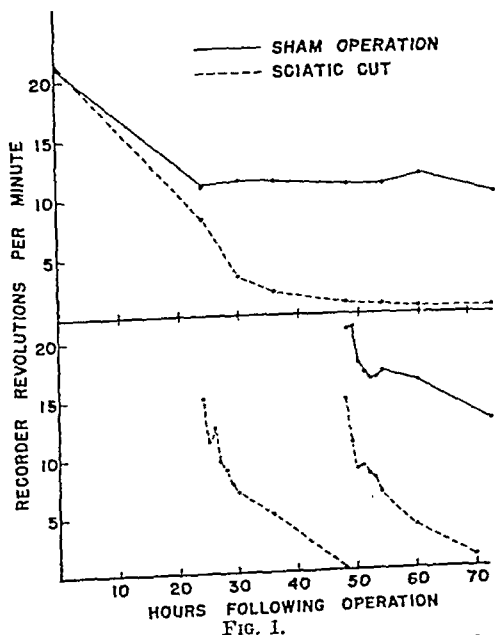


FIG. 1.
The effect of cutting the sciatic nerve upon the ability of the gastrocnemius muscle to respond to faradic stimulation.

muscle. Although the response of muscle to direct stimulation has commonly been described as due to the direct excitability of muscle fibers, it seems possible that the spread of the electrical pulse through the entire muscle may excite the nerve fibers which innervate it to play an important role in eliciting the response of muscle.

Separate experiments (unpublished data) have been carried out to determine the effect of curare upon the ability of the muscle to sustain responsiveness to faradic stimulation. If it were possible to completely block the myoneural junction without unfavorable effects upon respiration and circulation, the structures involved in the response to the stimulus could be further elucidated. Normal rats were given continuous subcutaneous injections of curare for periods up to 48 hours. In these experiments curare did not affect work output in any dosage which did not cause respiratory failure. It was also shown that doses which failed to cause respiratory

symptoms also failed to completely block the myoneural junctions in the stimulated muscle.

Summary. Male rats (310 to 350 g) were subjected to direct faradic stimulation of the gastrocnemius muscle following the cutting of the sciatic nerve, and their work performance was compared to that of sham-operated controls. When stimulation was started immediately following the operation the rate of work of denervated muscle was normal for several hours but was depressed below normal by 24 hours and continued to decrease rapidly, whereas the control animals sustained a high rate of work. When stimulation was delayed for 24 and 48 hours following denervation, the initial contractions were subnormal and decreased rapidly during continued stimulation. When the intensity of the pulse was increased from 12 to 20 milliamperes, at the end of each experiment there was a striking recovery in the rate of work of denervated muscle but it remained below that of the sham-operated controls.

16238 P

Rodenticidal Action of 2-Chloro-4-Dimethylamino-6-Methylpyrimidine (Castrix).*

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At the close of the European phase of the war it was revealed that German scientists had developed a new rodenticide (2-chloro-4-dimethylamino-6-methyl-pyrimidine) which was known as "Castrix." Since no data on the toxicity of Castrix were available in this country and no antidote against accidental poisoning in man and domestic animals was known the present investigation was under-

taken to ascertain the toxicity of Castrix to several species, to examine its efficacy as a rodenticide, and to find an effective antidote for the treatment of accidental poisoning. The results of this preliminary study demonstrated the high toxicity of Castrix and the effectiveness of pentobarbital sodium as an antidote against Castrix poisoning.

Experimental. In all of the species examined Castrix produced symptoms typical of central nervous system stimulants. Convulsions occurred after a latent period of 15 to 45 minutes following either oral or intraperitoneal administration. The head retracted and the fore-legs and hind-legs were tetanically extended. After the tonic phase

* The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Chicago Toxicity Laboratory. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

TABLE I.
Acute Toxicity of Castrix.

Species	Route of administration	LD ₅₀ ± S.E. (mg per kg)	No. of animals
Rat	Oral	1.25 ± 0.10	30
Rat	Intraperitoneal	1.00 ± 0.06	132
Mouse	"	0.42 ± 0.05	82
Guinea pig	"	2.66 ± 0.10	65
Rabbit	"	ca. 5	13
Dog	"	ca. 0.5	8

clonic movements spread over the entire body increasing in intensity for several seconds and then subsided. Several convulsive seizures occurred intermittently terminating with death after lethal doses or complete recovery after sublethal doses.

The high toxicity of Castrix[†] to 5 species is shown by the data in Table I. No differences in toxicity were observed when either aqueous or aqueous ethanol solutions (10% ethanol) were employed. A comparison of the toxicity of Castrix to 20 male and 20 female rats indicated that there was no sex difference in susceptibility. No evidence of tolerance was obtained when rats were given sublethal doses for varying periods and then given a lethal dose (2 mg/kg) of Castrix. The toxicity data shown in Table I were obtained during the summer months. Subsequent tests have shown some seasonal variation in the toxicity of this rodenticide to rats since the LD₅₀ of Castrix to Sprague-Dawley rats increased to 2.5 mg/kg during the winter months.

To test the acceptability of Castrix when offered to rats in the food 3 groups, each containing 5 rats, were fed Wayne Dog Blox *ad libitum* and were then offered the same diet ground and mixed with varying amounts of Castrix. The acceptability and high

lethality of Castrix offered in the diet are shown in Table II.

The symptoms of Castrix poisoning suggested that anti-convulsants might be effective antidotes. Sodium pentobarbital (Nembutal) was chosen for the present studies. Since convulsions constitute the first observable sign of poisoning, an antidote would have no practical value in man and domestic animals unless it were effective when administered during or after the onset of convulsions. Therefore, in these experiments Nembutal was never administered until the first convulsion began in about 25 minutes after administration of the rodenticide.

The data in Table III show the antidotal action of Nembutal in Castrix-poisoned rats. It is apparent from these results that at least 10 LD₅₀ doses of Castrix can be effectively counteracted by Nembutal. Since the animals which succumbed to 20 mg/kg of Castrix died in extreme central depression it is possible that more careful regulation of the dosage of the barbiturate might result in complete protection against 20 LD₅₀ doses.

Preliminary experiments with dogs have shown that Nembutal is effective in counteracting at least 15 to 20 LD₅₀ doses of Castrix. Since it was necessary to prolong the treatment for at least 13 hours when 10 mg/kg were given to dogs it appears that Castrix is detoxified more slowly in dogs than in rats.

Summary and Conclusions. A powerful convulsive agent, Castrix (2-chloro-4-dimethylamino-6-methylpyrimidine) is more toxic to rats than is either alpha-naphthylthiourea¹ or sodium fluoroacetate² by oral

TABLE II.
Acceptability of Castrix to Rats.

% Castrix in diet	Mortality	Time of death (hr)
1.00	5/5	2
0.50	5/5	<12
0.25	5/5	<12

[†] We are indebted to Dr. J. R. Stevens of the Baker Chemical Co., Phillipsburg, N.J., for the Castrix used in these experiments.

¹ Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**, 927.

² Kalmbach, E. R., *Science*, 1945, **102**, 232.

TABLE III.
Treatment of Castrix Poisoning in Rats with Sodium Pentobarbital.

Dose of Castrix (mg/kg)	No. of rats	Pentobarbital initial dose (mg/kg)	Schedule of pentobarbital treatment Hr after Castrix				% survival
			1	1.5-2	3	6	
5	5	45	30	20			100
5	5	45	30		20		100
10	5	45	30	30		30	100
20	5	30	30		15		60
20	5	30		30	20	15	60

and parenteral administration. Diets containing 0.25% to 1% of Castrix are readily eaten by rats and are highly toxic. No perceptible sex difference in susceptibility and no noticeable tolerance were observed in rats but some

seasonal variation in susceptibility was noted. Sodium pentobarbital is an effective antidote against at least 10 LD₅₀ doses of Castrix in rats and dogs even when administered after convulsions have begun.

16239

Lack of a Hypoglycemic Response to Intrathecal Injection of Glucose.*

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Marinelli and Giunti have recently presented data which clearly indicated that an increase in the glucose concentration of the spinal fluid of dog and man induced a rapid and pronounced reduction in the peripheral blood sugar level.¹ Lackey failed to obtain similar results in dogs.² In view of the paucity of evidence concerning the mechanisms whereby the central nervous system exerts its influence on the regulation of the blood sugar level, it was considered advisable to attempt to confirm the observations of Marinelli and Giunti in man. Consequently, we studied the influence of an increase in the cerebrospinal fluid glucose concentration on the blood sugar level.

Methods. Subjects suffering from a variety

of cerebrovascular and psychiatric disorders were studied after a preliminary fast of approximately 12 hours. Samples of blood were obtained from the antecubital vein before and at intervals after the intrathecal injection of glucose. Spinal fluid samples were obtained throughout the period of observation by means of an inlying needle inserted into the lumbar subarachnoid space. Glucose was injected into the spinal fluid in quantities of 5 to 10 ml of a 5% sterile solution in distilled water (250-500 mg) by way of either the cisternal or lumbar routes. The glucose concentrations of both blood and spinal fluid were determined by the Nelson modification³ of the Somogyi method.

Results. The data obtained in this study are listed in Table I. In no instance was there noted any significant change in the blood sugar level in spite of the marked increase in the cerebrospinal fluid glucose content. It will

* Aided in part by Research Grant No. 917 of the Research Grants Division, U. S. Public Health Service.

¹ Marinelli, L., and Giunti, V., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 23.

² Lackey, R. W., *Science*, 1947, **106**, 618.

³ Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

TABLE I.
Blood and Spinal Fluid Sugar Levels After Intracisternal Injection of Glucose in Man.

Subject	Glucose injected into cisterna mg	Spinal fluid* sugar—mg %				
		Before	15 min.	30 min.	45 min.	60 min.
1	250	62	70	63	64	61
2	250	76				630
3	400	57	1011	913	832	830
4	500	60	239	406	761	126
5	500	60	118	80	76	97
6	500	66	65	70	84	736
7	500	72	536	736	782	767
8	500	56	208	638	742	

Blood sugar—mg %					
Before	15 min.	30 min.	45 min.	60 min.	
1	85	82	83	81	76
2	88	96	94	97	104
3	72	68	70		71
4	76	76	71	73	74
5	86	90	88	86	86
6	79	85	83	82	82
7	98	95	100	97	100
8	80	85	91	98	81

* Drawn from L4.

also be noted that the intracisternal injection of glucose was followed by a marked rise within 15 minutes in the glucose content of spinal fluid withdrawn from the lumbar region. Such a finding indicates the free diffusion of the injected glucose and may be taken as presumptive evidence that every part of the central nervous system was in contact with an increased concentration of glucose.

Since similar results have been obtained in subjects free from neurologic and psychiatric disorders, it is obvious that an increase in

the glucose level of the cerebrospinal fluid does not initiate a chain of reactions resulting in a decrease of the blood sugar level. It seems fairly definite, therefore, that any mechanisms that may be involved in the regulation of carbohydrate metabolism by the central nervous system are not significantly influenced by increase in the glucose content of the spinal fluid.

Summary and Conclusion. Increases in the spinal fluid glucose concentration do not produce any effect on the blood sugar level.

16240

Infectivity of Sporozoites of *Plasmodium Cathemerium* 3H2 Exposed *In Vitro* to Hen and Canary Bloods.*

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In this laboratory the avian malarial parasite, *Plasmodium cathemerium* 3H2, has been maintained by continuous mosquito-canary-

* This research was supported in part by a grant from the National Institute of Health.

mosquito passage since February, 1937. During all of this time there has never been a failure to infect a canary; *i. e.*, in the rare instances in which a first trial has not succeeded a second trial has always done so. But

it has not been possible to infect with this organism the great horned owl,¹ the guinea pig,² the barnyard hen, or man.³ In this present communication there will be recorded the results of an attempt to determine whether the blood of the hen contains a factor inhibitory to the sporozoites of *P. cathemerium* 3H2 when they are exposed to it *in vitro*. For the purposes of the study the sporozoites were incubated separately but simultaneously for $\frac{1}{2}$ to $2\frac{1}{2}$ hours in hen and canary bloods, and then the blood-sporozoite mixtures were injected intramuscularly into susceptible canaries.

Method. Using a tuberculin syringe to which was attached a 24-gauge $\frac{3}{4}$ -inch needle filled with 1% sodium heparin solution preserved with 0.5% phenol, 0.2 cc of blood was drawn from the heart of each of 8 canaries to make a total of 1.6 cc. The blood was pooled as drawn in a rubber-stoppered 5-cc vaccine bottle held in the mouth of the operator and kept in motion by a frequent bobbing of the head, both movement of the blood and fair maintenance of temperature being accomplished by this maneuver. The blood was drawn in about 10 minutes total elapsed time and the bottle was then immediately placed in an incubator at 41.5°C, being affixed in the incubator to a rotor making 1 complete revolution per minute. Immediately after bleeding the canaries, an amount of blood—7 cc—thought to be equivalent to the fraction of the total drawn from the canary was taken by heart puncture from each of 3 hens, using a 3-inch "Bishop B-19" needle containing heparin solution in amount proportionate to that employed in bleeding the canaries. At once, 0.5 cc of each of 2 of these hen blood samples and 0.6 cc of the third were placed together in a 5-cc vaccine bottle, making a total of 1.6 cc as in the case of the canary blood. The pool of hen blood was usually prepared and in the incubator rotor in 5 to 8 minutes. Then as quickly as possible the

sporozoites were prepared for addition to the bloods, as follows: 100 *Culex pipiens* mosquitoes that had taken an infective blood meal 2 weeks previously were stunned by rapping the Kjeldahl flask in which they were contained a number of times against the palm of the hand, after which they were dumped into a small evaporating dish containing 1.0 cc of Locke's solution. With a fairly large pestle the mosquitoes were then ground for precisely 30 seconds; the triturate was then transferred to 3 layers of gauze in a small funnel protruding through a stopper into a calibrated centrifuge tube and washed through the gauze with sufficient Locke's solution to bring the filtrate up to the 1.0 cc mark in the tube. Then the bottles of blood were taken out of the incubator and 0.4 cc of the sporozoite-containing filtrate, withdrawn from the centrifuge tube through a specially devised long needle with a square end and a very fine bore, was added to each. The bottles, which now contained 2 cc of a sporozoite-blood mixture, were at once returned to the incubator and rotation resumed. Thereafter, at 30-minute intervals through $2\frac{1}{2}$ hours, the bottles were taken from the incubator just long enough to withdraw 0.2 cc of the contents of each into separate tuberculin syringes, having 27-gauge $\frac{1}{2}$ -inch needles attached. At each withdrawal period 4 clean canaries were injected intramuscularly with the canary blood-sporozoite mixture and 4 with the hen blood-sporozoite mixture; each bird received 0.05 cc, an amount calculated to contain the sporozoites from 1 mosquito. The peripheral blood of the injected canaries was examined for the presence of plasmodia on the 6th to 16th days, inclusive, following the injections, and thereafter any canaries in whose blood organisms had not appeared were challenged by the bites of infected mosquitoes to determine whether or not they were still susceptible to malarial infection.

Results. Tables I and II comprise the findings in this study. The 4 separate trials were made a sufficient number of weeks apart to permit a full cycle of events to have transpired in the insectory so that each trial was done with a new and freshly infected lot of mosquitoes. In Table I there is ex-

¹ Beekman, H., and Ota, R. K., *Proc. Soc. Exp. Biol. and Med.*, 1940, 45, 477.

² Beekman, H., and Smith, J., unpublished findings.

³ Beekman, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, 60, 401.

TABLE I.
Infectivity of Sporozoites of *P. cathemerium* 3H2 Exposed *in vitro* to Hen and Canary Bloods. (Data from a Single Trial.)

Exposure period (hr)	Type of blood	Bird No.	First day of positive blood	Type of blood	Bird No.	First day of positive blood	Result of mosquito challenge
$\frac{1}{2}$	canary	1605	9	hen	1497	None	Infected
	"	1126	9	"	1665	"	"
	"	1843	8	"	1983	"	"
	"	1821	8	"	1777	"	"
1	"	1579	11	"	822	"	"
	"	1171	10	"	817	"	"
	"	1482	10	"	1141	"	"
	"	69	8	"	1024	"	"
$1\frac{1}{2}$	"	1827	10	"	1856	"	"
	"	1679	10	"	801	"	"
	"	1637	8	"	850	"	"
	"	1713	9	"	961	"	"
2	"	91	10	"	840	"	"
	"	1086	12	"	1073	"	"
	"	1642	9	"	1956	"	"
	"	818	11	"	878	"	"
$2\frac{1}{2}$	"	61	9	"	1563	"	"
	"	1144	13	"	1096*	"	"
	"	1604	10	"	1555	"	"
	"	831	11	"	76	"	"
Totals:	Birds injected	20		Birds injected	19		All infected
	" infected	20		" infected	0		

* Traumatic death on day of injection.

hibited the data from a single trial; it will be noted that organisms were found in the peripheral blood in 8 to 13 days in all of the birds injected with sporozoites in canary blood and that no organisms were found at any time in those injected with sporozoites in hen blood; it will be further observed that all of the latter group of birds did reveal organisms in the peripheral blood when they were subsequently bitten by infected mosquitoes. In Table II, comprising a summary of all the trials, it will be seen that of the 80 birds injected with sporozoites in canary blood 97.5% became infected, while of the 76 birds injected with sporozoites in hen blood only 2.6% became infected; it will also be noted that all 74 of the latter group of birds became infected when subsequently challenged by infected mosquitoes.

Discussion. The findings in the present study establish the fact that the blood of the hen, an animal not susceptible to infection with *P. cathemerium* 3H2, exerts an inhibit-

ing action upon the sporozoites of this organism *in vitro*. Coggeshall,⁴ stating that there is considerable experimental evidence in support of the assumption that an immune serum can act upon a parasite without the participation of the cells of the host, showed that some sera from monkeys that had survived a *P. knowlesi* infection completely inhibited the infectiousness of the erythrocytic form of the parasite when it was incubated with them for $\frac{1}{2}$ hour. He showed that the protective antibodies in such a serum can be removed by absorbing them by the addition of living parasites and then removing both by centrifugation, but his experimental arrangement did not lend itself to a study of the effect of these sera upon sporozoites. It seems from the work of Boyd⁵ that the immune mechanism engendered by an attack of vivax malaria in the human is specifically directed toward the trophozoites

⁴ Coggeshall, L. T., *Medicine*, 1943, 22, 87.

⁵ Boyd, M. F., *J. Nat. Med. Soc.*, 1947, 6, 12.

TABLE II.

TABLE II.
Infectivity of Sporozoites of *P. cathemerium* 3H2 Exposed *in vitro* to Hen and Canary Bloods.
(Summary of all trials.)

	Canary blood		Hen blood		
Trial No.	Birds injected	Birds infected	Birds injected	Birds infected	Result of mosquito challenge
One-half Hour Exposure.					
1	4	4	2*	0	2 infected
2	4	4	4	0	4 "
3	4	4	4	0	4 "
4	4	4	3*	2	1 "
One Hour Exposure.					
1	4	3	4	0	4 "
2	4	4	4	0	4 "
3	4	4	4	0	4 "
4	4	4	4	0	4 "
One and One-half Hours Exposure.					
1	4	4	4	0	4 "
2	4	4	4	0	4 "
3	4	4	4	0	4 "
4	4	4	4	0	4 "
Two Hours Exposure.					
1	4	4	4	0	4 "
2	4	4	4	0	4 "
3	4	3	4	0	4 "
4	4	4	4	0	4 "
Two and One-half Hours Exposure.					
1	4	4	4	0	4 "
2	4	4	3*	0	3 "
3	4	4	4	0	4 "
4	4	4	4	0	4 "
Totals	80	78	76	2	74 "
Percentage infected after exposure to canary blood				97.5	
" " " " " hen				2.6	

* Numbers less than 4 reflect early deaths from injection trauma.

(i. e., the erythrocyte phase) and not toward the sporozoites. Mulligan, Russell and Mohan⁶ reported that the sera of many species of normal animals and man agglutinate the sporozoites of *P. gallinaceum* in low dilutions but that agglutination in high dilutions occurred with only the sera of animals or men having chronic malarious infections. It was not determined in the present study whether the effect of the hen's blood upon the sporozoites was an agglutinative one. Huff and Coulston,⁷ failing to find cryptozoites in canaries inoculated with the sporozoites of *P. gallinaceum*, made the assumption that the barrier must exist in macrophages and other phagocytic cells or in antagonistic humoral

substances. Since in the present study it is shown that canaries injected with sporozoites that have been exposed to hen blood not only fail to become infected but can later be infected by sporozoites that have not had such contact, it seems likely that hen's blood depresses the sporozoites sufficiently to prevent the initiation by them of the exoerythrocytic cycle.

Summary. Canaries were injected intramuscularly with sporozoites of *P. cathemerium* 3H2 in pooled hen or canary blood, the sporozoite-blood mixtures having been incubated previously at 41.5°C, with agitation, for periods of ½, 1, 1½, 2, and 2½ hours. Of 80 canaries injected with sporozoites in canary blood, 97.5% became infected; of 76 canaries injected with sporozoites in hen blood, 2.6% became infected. All 74 canaries which failed to become infected when injected with

⁶ Mulligan, H. W., Russell, P. F., and Mohan, B. N., *J. Mal. Inst. India*, 1940, **3**, 513.

7 Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1946, 78, 93.

sporozoites in hen blood did become infected when subsequently bitten by infected mosquitoes. It is felt that these findings suggest the existence of a factor in the blood of the hen that prevents the initiation by the sporozoite of the exoerythrocytic cycle in the canary.

Subsequent note: Further studies, com-

pleted after the above manuscript was submitted for publication, have revealed that (a) the inhibitory action of hen's blood on the sporozoites is exerted though sodium citrate is used as anticoagulant instead of heparin; and (b) it is not lessened by agitating the blood at room temperature for an hour before adding the sporozoites to it.

16241 P

Activity of Hexokinase Preparations from Rat Muscle.*

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Hexokinase activity involves the conversion of an easily hydrolyzable phosphate group to one not easily hydrolyzable (glucose-6-phosphate). Since the P_7^{\dagger} value of the reaction mixture includes both the P_0^{\dagger} and the $\Delta 7P^{\dagger}$ but not the P in glucose-6-phosphate, any conversion of $\Delta 7P$ to glucose-6-phosphate will be reflected in a decrease in the P_7 values during incubation. However, the calculation of hexokinase activity in complex systems by means of such changes poses a difficult problem since transphosphorylation from ATP may fail to account for all of the phosphate changes actually occurring.

In rat muscle preparations at least two other types of transfer, in addition to transphosphorylation, are often observed. Large increases in inorganic phosphate may occur, even in the presence of fluoride, due to the dephosphorylation of ATP by the apyrases¹ present in such preparations. In addition

part of the inorganic phosphate formed during incubation may be derived from sources other than the $\Delta 7P$. The action of the apyrases is accompanied by a large increase in the P_0 content, a decrease in the $\Delta 7P$ and no change in the P_7 value. The increase in inorganic phosphate from extraneous reactions is manifested by increases in both the P_7 and P_0 values. The increase in the P_7 value indicates that fraction of the total increase in inorganic phosphate that is attributable to the dephosphorylation of esters other than those containing easily hydrolyzable phosphate.

In order to demonstrate hexokinase activity by means of phosphorous transfers, it is essential to compare the changes occurring during incubation in the absence and presence of glucose. If the addition of glucose failed to influence phosphate changes other than those occurring as the result of the transphosphorylation catalyzed by hexokinase, calculation of the activity from a determination of the effect of glucose on the easily hydrolyzable phosphate content would be entirely feasible.² There is good evidence, however, that the presence of glucose may influence other reactions. Meyerhof and Geliaskowa³ demon-

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[†] The following symbols are used: P_0 —inorganic phosphate determined prior to hydrolysis; P_7 —inorganic phosphate content determined after 7 minutes' hydrolysis at 100°C in 1 N HCl; $\Delta 7P$ —that fraction of esterified phosphate converted to inorganic phosphate by 7 minutes' hydrolysis at 100°C in 1 N HCl (i. e., $P_7 - P_0$).

¹ Meyerhof, O., *J. Biol. Chem.*, 1945, **157**, 105.

² Colowick, S. P., and Price, W. H., *J. Biol. Chem.*, 1945, **157**, 415.

³ Meyerhof, O., and Geliaskowa, N., *Arch. Biochem.*, 1947, **12**, 405.

TABLE I.

Calculation of Hexokinase Activity in Extracts and Homogenates of Rat Muscle.
 Each flask contained the following components: Side arm—0.1 ml 0.05 M or 0.1 M Na-ATP (pH 7.5), 0.15 ml 0.9 M NaF and 0.05 M NaHCO₃, 0.1 ml 1% glucose or water. Main compartment—0.4 ml 0.15 M NaHCO₃, 0.4 ml 0.05 M MgCl₂, 0.5 ml extract or homogenate and H₂O to 2.05 ml. Incubation for 30 min. in 65% N₂, 5% CO₂ at 30°C. All values are expressed in terms of μg per ml of reaction mixture. The examples given are typical of a great number of similar results.

Preparations	Glucose	Before incubation				After incubation				Hexokinase activity expressed as decrease in P_7 during incubation		Hexokinase activity expressed as decrease in P_7 during incubation	
		P_0		P_7		P_0		P_7		$(B) - (A)$	$(B) \cdot (A)$	$(B) - (A)$	$(B) \cdot (A)$
		P_0	P_7	P_7	P_0	P_0	P_7	P_7	P_0				
Extract	(A)	104	231	127	127	127	235	108	108	—4	19	19	96
	Present (B)	104	231	127	127	104	186	82	82	45	45	45	45
Homogenate	(A)	119	440	321	321	230	458	228	228	—18	93	93	19
	Present (B)	119	440	321	321	217	426	209	209	14	32	112	112

strated that, in both homogenates and extracts of brain tissue, dephosphorylation of ATP occurs to a much greater degree in the absence than in the presence of glucose. We⁴ reported similar findings in the case of homogenates and extracts obtained from rat muscle. In the presence of fluorides the activity of the apyrases although markedly inhibited still remains appreciable. The inclusion of glucose in the reaction mixture enables the hexokinase reaction in extracts to proceed through its successful competition with the apyrases for the participation of ATP. Obviously, since the presence of glucose results in an inhibition of apyrase activity, calculation of the hexokinase effect by a comparison of the changes in the $\Delta 7P$ after incubation in the absence and presence of glucose represents the summation of hexokinase and apyrase activity. On the other hand, although apyrase activity is accompanied by a decrease in the $\Delta 7P$ and an increase in the P_0 , it fails to influence the P_7 values. It is therefore perfectly justifiable to calculate hexokinase activity from a comparison of the P_7 changes during incubation in the presence and absence of glucose provided that these two reactions are the only ones occurring which involve phosphate transfers to an appreciable degree.

As a matter of fact, in rat muscle extracts of the type described by Colowick, Cori, and Slein,⁵ the sole phosphate changes observed to any degree are those due to these two reactions. In homogenates, on the other hand, extraneous phosphate formation is also observed. Since this latter type of transfer involves changes in both the P_7 and P_0 values, calculation and demonstration of hexokinase activity in homogenates forms an almost insoluble problem.

Since the method of obtaining hexokinase-containing extracts, as described by Colowick *et al.*,⁵ yields preparations which fail to present such complex problems, it is obvious that this type of extract should preferably be employed for the demonstration of activity.

⁴ Broh-Kahn, R. H., and Mirsky, I. A., *Fed. Proc.*, 1947, 6, 83.

⁵ Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, 168, 553.

In view of the considerations noted above, attempts to estimate hexokinase activity in homogenates may be of little avail in spite of the fact that such preparations may contain large amounts of the desired system. The data in Table I illustrate these points.

Summary. The activity of apyrase systems and extraneous phosphate formation

interfere with the determination of hexokinase activity in rat muscle homogenates. Hexokinase activity in rat muscle extracts is best calculated from changes in the P_i values during incubation, a procedure which avoids the complication of the inhibition of apyrases by hexokinase activity.

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Anemia in Rats Infested with *Bartonella muris* and Injected with Pteroylglutamic Acid.*

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An active infestation of *Bartonella muris* in rats leads to an acute anemia, hemoglobinuria, emaciation, and usually death.¹⁻⁴ In our experience the number that die may be reduced somewhat by injecting the infested blood into the rat several days or weeks before the spleen is removed rather than giving it immediately after splenectomy. Death rate has varied considerably in our rats, being around 50% a few years ago^{4,5} and about 25% in the present study. Several efforts have been made to discover a satisfactory treatment for this disease. Some substances such as arsenicals,⁶ extracts of spleen, copper, and iron,⁷ have been found to be beneficial. Negative results were obtained in the treat-

ment of the infestation with sulfanilamide.⁴

Since pteroylglutamic acid (PGA, folic acid) was found to be helpful in anemias of different types^{8,9} it was decided to try it in rats made anemic by *Bartonella muris* infestation.

The rats used in these experiments were nearly full grown albino males fed on Purina laboratory chow (containing 2.4 μ g of PGA per g).[†] They ate approximately 15 g per day at the start and much less during the severe anemia. They were injected intraperitoneally, with saline containing blood taken from rats suffering from acute infestation of *Bartonella muris*. Several days to weeks later they were prepared for operation, etherized and the spleen removed. Hemoglobin estimations and red cell counts were done every 3 to 6 days for several weeks. The hemoglobin was determined by the Sahli method. These readings were checked with the photoelectric acid hematin method for hemoglobin and found to be consistently only slightly higher. The Sahli method was there-

* Research Paper No. 876, Journal Series, University of Arkansas.

¹ Ford, W. W., and Eliot, C. P., *J. Exp. Med.*, 1928, **48**, 475.

² McCluskie, J. A. W., and Niven, Janet, *J. Path. and Bact.*, 1934, **39**, 185.

³ Weinman, D., *J. Infect. Dis.*, 1938, **63**, 1.

⁴ Emery, F. E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 56.

⁵ Emery, F. E., Bugelski, T. S., and Schwabe, E. L., *Endocrinology*, 1940, **26**, 167.

⁶ Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Wschr.*, 1926, **5**, 559.

⁷ Perla, D., and Marmorston-Gottesman, J., *J. Exp. Med.*, 1932, **56**, 777, 783.

⁸ Higgins, G. M., *Proc. Staff Meet. Mayo Clin.*, 1944, **19**, 329.

⁹ Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 604.

[†] We are indebted to Mrs. Edith S. Sims for these determinations and for the PGA solutions used.

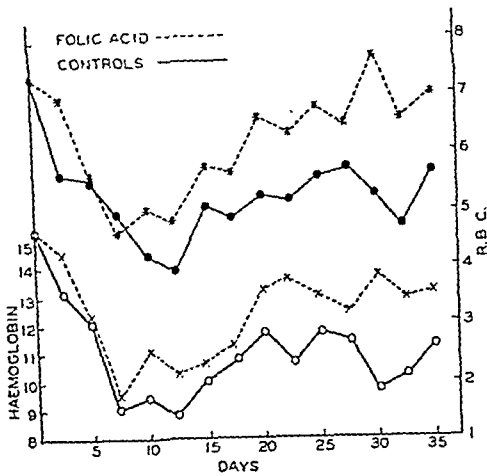


FIG. 1.

Two lower curves hemoglobin in grams of splenectomized rats infested with *Bartonella muris*. Mean values for twenty animals included in each curve. Two upper curves red blood cells in millions.

fore considered to be very satisfactory for these experiments.

Some of the rats in each group were given daily intramuscular injections of 40 μ g of pteroylglutamic acid. The means (for 20 animals in each group) at intervals of 2½ days, arranged as curves of hemoglobin in grams and red cell counts in millions, are shown in Fig. 1. It will be noted that the spread between the treated and control groups is narrow for about 18 days and widens considerably thereafter. This is taken to indicate that pteroylglutamic acid aids new cell formation and in this way keeps the blood count up above the untreated rats during the progressive anemia of the *Bartonella* infestation; after the anemia has reached its lowest level, and blood regeneration exceeds red cell breakdown, then the injected animals have considerable more capacity for new red cell formation than the controls as the curves clearly show. This gives rise to analysis of the curve in two parts. The mean for all the hemoglobin determinations during the first 18 days

in the injected rats has been compared with the mean hemoglobin content of the untreated rats and found to give a mean difference which is very significant, being more than 5 times the standard error of the difference. A similar comparison from the nineteenth to thirty-fifth day showed the mean difference in hemoglobin at this end of the curve to be over 10 times the standard error of the difference. Likewise the red cell count is also significantly higher in the rats treated with pteroylglutamic acid and the results of the analysis for this data are similar to those described for hemoglobin.

From these data it is obvious that pteroylglutamic acid is beneficial in holding the hemoglobin and numbers of red cells in the treated rats above those of the controls. From Fig. 1 it may be seen that the curves for the injected rats are above the controls as early as 2½ days after splenectomy and after injections of pteroylglutamic acid were started. The data are not numerous enough to establish a significant difference between the curves at this point. If the curves are different as early as 2½ days it would be interesting information on the function of pteroylglutamic acid and may suggest a role aside from red cell formation. On the other hand the formation of new cells in numbers sufficient to be detected in the general circulation might occur in a few days in these anemic rats. Since there was some PGA in the food these results would probably be all the more striking in rats on a PGA-free diet.

Summary. Hemoglobin and red cell estimations have been done on the blood of rats made anemic by splenectomy and *Bartonella muris* infestation. Animals injected with pteroylglutamic acid show hemoglobin and red cells at higher levels than those of the untreated controls. Curves made from this data show the treated animals much less anemic than the controls for a period of 35 days or longer.

Do Chromosomes Manifest Osmotic Volume Change?

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In an earlier study¹ it was shown that the nucleus of the immature starfish egg in the germinal vesicle stage undergoes a kinetics of swelling and shrinking which is formally similar to that of the egg as a whole. Observations made also on equilibrium volumes indicated that the germinal vesicle contains a smaller percentage of osmotically inactive material than does the cytoplasm in this cell. The advantage of studying the nucleus in the germinal vesicle stage is that its size is large enough to realize sufficiently accurate diameter measurements under high dry magnification. When the egg matures, the nucleus becomes very much smaller, and direct measurements are rendered considerably more difficult by this method.

In the spermatozoon, the chromosomes are compressed into the smallest possible volume in an animal cell, nearly devoid of cytoplasm, hence an examination of the osmotic properties of the sperm affords the nearest approach to an intracellular study of the osmotic properties of the chromosomes themselves, in the intact living cell. Since spermatozoa are not spherical, and moreover are so small, it is necessary to use some method other than direct microscopic measurement, such as centrifuging, for estimation of cell volume. Apparently the first to study this quantitatively (though not with reference to the question of chromosome swelling) was Hamburger² who obtained an average value of 72.8% for the "Gerüstvolum" (literally, "framework volume": operationally, the equivalent of "osmot-

ically inactive fraction"³) of frog spermatozoa. In arriving at this value, he used low centrifugal forces (1800 or 3000 r.p.m., no radius stated) and placed the sperm in solutions of pure NaCl, a procedure known to be toxic to a variety of cells.⁴ It was therefore decided to investigate the matter in a species where sperm are readily available in abundance, using a balanced ionic environment and a centrifugal force adequate to pack the cells quantitatively, and to test the applicability of the Boyle-van't Hoff law.

According to Harvey and Anderson⁵ the *Arbacia* sperm has a pointed head with a flattened base (2 μ wide) adjacent to a short slightly narrower middle piece which seems to contain a pair of spherical bodies. The length of the head and middle piece is approximately 4 μ . The length of the tail is about 45 μ . Direct observations of swelling to about twice their original size were made of sperm placed in distilled water. Electron microscope observations revealed the tail to be composed of about 10 strands of uniform thickness, each about 50 m μ in diameter. Owing to the thickness of the heads, it was not possible to make out any discrete internal structure.

Procedure. Sperm were obtained from ripe testes of the sea urchin, *Arbacia punctulata*, by excision from the animal, and allowing the spermatid fluid to exude as a viscous creamy suspension into a stender dish. The material was pipetted into a test tube and centrifuged lightly in order to throw down any gross tissue particles. This still left the bulk of the sperm in suspension, occupying from 23.5 to 27.8% of the total volume. Hypotonic sea water solutions, varying in concentration from 1% to 80%, were made up fresh each day. Owing to the fact that the cell volume is only a fraction of the spermatid fluid, mixture with

1 Beek, L. V., and Shapiro, H., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 170.

2 Hamburger, H. J., *Osmotischer Druck und Ionenlehre*, 1904. Wiesbaden, J. F. Bergmann. Vol. 3. p. 4. Die Volumänderungen der Spermatozoen unter den Einfluss hypertotonischer und hypotonischer Salzlosung.

3 Lucké, B., and McCutcheon, M., *Physiol. Rev.*, 1932, **12**, 68.

4 Loeb, J., *Pflüger's Arch.*, 1903, **97**, 394.

5 Harvey, E. B., and Anderson, T. F., *Biol. Bull.*, 1943, **85**, 151.

the hypotonic sea waters yielded a range of resultant effective osmotic concentrations of 42.4 to 88.5% sea water.

The spermatid fluid was pipetted in 0.1 ml aliquots into small shell vials, an equal quantity of hypotonic sea water was added to each vial, and the contents mixed thoroughly. The shell vial was covered with a cover slip, and from 50 minutes to 2 hours allowed for the attainment of osmotic equilibrium at room temperature (about 25°C). Specially drawn capillary tubing was used, of uniform bore (approx. 0.39 mm, checked by microscope measurements of diameters at both ends) and outside diameter, 0.56 mm. The tubing was cut up into lengths of about 43 mm, and from 30 to 35 mm of diluted sperm suspension drawn up by capillarity, following which the opposite end was sealed off in a microflame. The perfection of each seal was checked by microscopic examination. Centrifuging (15 to 17 minutes duration) was carried out in an air turbine, at approximately 20,000 times gravity, in a steel rotor made available through the courtesy of Dr. A. K. Parpart. Column lengths were measured under a binocular dissecting microscope with the aid of a metric steel rule. At this centrifugal force erythrocytes are completely packed; for *Arbacia* eggs it has been estimated that a minimal centrifugal force of $4330 \times$ gravity is required to pack the cells completely.⁶ The exact force at which complete packing is attained will be related to the physical properties of the cell in question, and to the medium.⁷

Condition of the Sperm. The physiological state of the sperm cells was studied after swelling by microscopic observation of their motility and of fertilizing power. In all the dilutions employed, numerous highly active sperm were observed. As the packed sperm were allowed to rest in the capillary tube after centrifuging, the cell column length increased slightly with time, apparently due to sperm at the free end of the column migrating into the supernatant fluid. When swollen sperm were returned to a suspension of eggs in normal sea water, high percentages of mem-

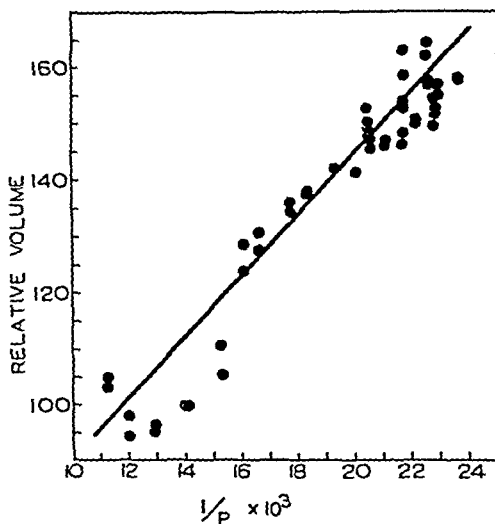


FIG. 1.
Showing the applicability of the Boyle-van't Hoff law to equilibrium volumes of sea urchin sperm swollen in various dilutions of sea water.

brane formation and cleavage ensued, demonstrating that the sperm had not been irreversibly affected by the swelling process.

Results. Fifty-six determinations were carried out, the results of which are summarized in Fig. 1, which is a plot of relative sperm volume against the reciprocal of the osmotic pressure of the medium. A rectilinear relation indicates the applicability of the Boyle-van't Hoff law,

$$P(V - b) = k,$$

where P , V , b , and k represent respectively osmotic pressure, cell volume, osmotically inactive fraction, and a constant. Extrapolation of the curve to the zero ordinate (not shown) yields an average value for " b ," the "osmotically inactive fraction," of 33%, a value in the same region as that observed for other cells (e. g., in unfertilized *Chaetopterus* eggs, " b " is 38.8%.⁸) The best average curve, fitted visually, has been drawn through the data. Application of the Criterion of Least Squares shows that the line so drawn lies close to one computed by least squares. Although the plotted experimental points in Fig. 1 show a fair amount of scatter, it is essential to note that if lines with the greatest

⁶ Shapiro, H., *Biol. Bull.*, 1935, 68, 363.

⁷ Cf., for example, Johnson, F. H., and Harvey, E. N., *J. Cell. and Comp. Physiol.*, 1937, 9, 363.

⁸ Shapiro, H., *J. Cell. and Comp. Physiol.*, 1941, 18, 143.

and least slope reasonably possible be drawn that "b" will have a maximum possible value of approx. 48%, and a minimum value of approx. 18%.

Discussion. Since the chromosomes appear to constitute the bulk of the content of the spermatozoon, it appears from this result that they must partake in the osmotic swelling of the cell. If the chromosomes did not swell, the value obtained for "b" would be very high, of the order of 90% or more, judging from the standard cytological pictures of sperm heads. In so reacting to hypotonic solutions, the chromosomes display an osmotic pattern shared by other formed elements within the nucleus and the cytoplasm, viz., the nucleolus, and the diverse types of granules which undergo osmotic volume changes. The value of 72% obtained by Hamburger for frog sperm may be due to a species difference or to the differing circumstances, alluded to in the introduction, in which the experimental determinations were carried out.

Churney⁹ studied the swelling of the germinal vesicle in immature eggs of *Arbacia punctulata* and *Nereis limbata*. Unfortun-

ately, no calculations of osmotically inactive fraction were given. He concluded that "the experiments failed to reveal any appreciable amount of osmotically inactive material in either of the nuclei." An interesting observation was also briefly reported in a footnote to the effect that "the giant chromosomes of the salivary gland cells of *Sciara coprophila* and of *Chironomus* sp. swell and shrink reversibly in anisotonic solutions." Quantitative data were not furnished.

Summary. A. The applicability of the Boyle-van't Hoff law to the swelling of spermatozoa of the sea urchin, *Arbacia punctulata*, in hypotonic solutions of effective concentrations varying from 42.4 to 88.5% sea water was investigated. Cell volumes were determined by centrifuging in small capillaries in an air turbine at 20,000 × gravity.

B. In the range of dilutions employed, the Boyle-van't Hoff law appeared to be followed. The "osmotically inactive fraction" ("b") averaged 33%. Thus the sperm head, which is chiefly chromosomal material, has a non-swelling volume which does not differ greatly from that of certain other cells. The results indicate that the chromosomes take part in the osmotic swelling of the cell.

⁹ Churney, L., *Biol. Bull.*, 1942, **82**, 52.

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An Investigation of Transmethylation from N¹-Methylnicotinamide

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With the discovery that nicotinamide is methylated in the animal body to give N¹-methylnicotinamide¹ it became of interest to know whether this product is a methyl donor in the biological synthesis of N- and S-methyl compounds. One approach to this problem has been the study of the lipotropic activity of N¹-methylnicotinamide when added to a methyl-deficient diet. Najjar and co-

workers^{2,3} have presented data to show that N¹-methylnicotinamide has some lipotropic action in the rat, whereas Handler and Dubin⁴ found in a similar experiment that it had no activity in preventing the development of fatty livers.

We wish to report here an experiment of

² Najjar, V. A., and Deal, C. C., *J. Biol. Chem.*, 1946, **162**, 741.

³ Najjar, V. A., and Ratcliffe, I. M., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 142.

⁴ Handler, P., and Dubin, I. N., *J. Nutrition*, 1946, **31**, 141.

* Present address: School of Biological Sciences, The University of Tennessee, Memphis, Tennessee.

¹ Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1943, **150**, 395.

TABLE I.

Feeding Experiment with N¹-deuteriomethylnicotinamide (86.0 Atom % D in the Methyl Group).

Rat No., Sex	Change in body wt g	Total amt of test compound ingested mg	Deuterium content of isolated compounds	
			Choline chloro-platinate atom %	Creatinine potassium picrate atom %
13 ♂	76-86	720	0.00 ± .09	0.00 ± .07
14 ♂	79-94	840	0.06 ± .13	0.00 ± .06

direct nature testing whether N¹-methylnicotinamide can function as a methyl donor in the body by labeling the methyl group of this compound with deuterium. N¹-deuteriomethylnicotinamide chloride was synthesized and fed to 2 growing rats for 6 days. At the end of this time the rats were killed and the body choline and creatine isolated. Analysis of the isolated compounds showed that no deuterium was present indicating that no transmethylation from N¹-methylnicotinamide to choline or creatine had occurred.

Experimental. Synthesis of N¹-deuteriomethylnicotinamide chloride (CD₃N(Cl)-C₅H₄CONH₂). This compound was prepared from deuteriomethyl iodide⁵ and nicotinamide by the method of Karrer *et al.*⁶ Two preparations were made, and weighed amounts of the two were mixed for the feeding experiment. For the determination of the deuterium content, the compound was burned, the water collected, purified, and the D₂O:H₂O ratio therein was measured by the falling drop method.⁷ From the results obtained it was calculated that the compound contained 86.0 atom % deuterium in the methyl group.

Analysis. N¹-deuteriomethylnicotinamide chloride.

Preparation 1. Calculated,† C 48.0; found, C. 47.9.

Preparation 2. Calculated, C 48.0; found, C 47.7.

⁵ du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, 1941, **140**, 625.

⁶ Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U., *Helv. Chim. Acta*, 1936, **19**, 811.

⁷ Cohn, M., in Wilson, D. W., *Preparation and Measurement of Isotopic Tracers*, J. W. Edwards, Ann Arbor, 1946, p. 51.

† Calculated values are based on an increased molecular weight due to the deuterium in the molecule.

Feeding Experiment. Two young litter-mate male rats were used. During a control period of 3 days they were fed *ad libitum* a diet of the following percentage composition: casein 18, sucrose 58, hydrogenated vegetable oil 19, corn oil 1, Osborne and Mendel salt mixture 4. In addition 100 g of the diet contained 20 mg choline chloride, 2 mg each of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and nicotinic acid, 4 mg calcium-*d*-pantothenate, 4000 U.S.P. units vitamin A, 400 U.S.P. units vitamin D, and 1 mg α -tocopherol acetate.

After the 3-day control period, 2% N¹-deuteriomethylnicotinamide chloride was put in the diet replacing an equivalent amount of sucrose. The 2 rats were pair fed. On the 6th day of feeding the test compound, rat 13 failed to eat. It was therefore killed, and autopsy showed that the kidneys were hemorrhagic. The kidneys of rat 14, which was killed at the same time, appeared to be normal. The changes in body weight and the amounts of N¹-deuteriomethylnicotinamide chloride ingested are given in Table I.

Creatine was isolated from the carcass of each rat as creatinine potassium picrate,⁵ the purity of which was determined colorimetrically by the Jaffé reaction. Choline was isolated as choline chloroplatinate.⁵

Analysis. Choline chloroplatinate.

Rat 13—Calculated, Pt. 31.7; found, Pt. 31.7.

Rat 14—Calculated, Pt. 31.7; found, Pt. 31.5.

The isolated compounds were analyzed for deuterium by the method mentioned above, and the values obtained are given in Table I.

Discussion. The isolated choline and creatine contained no deuterium. Thus, under the conditions of this experiment, there was no detectable transmethylation from N¹-methylnicotinamide to choline or creatine.

From the work of Handler and Dann,⁸ and Perlzweig, Bernheim, and Bernheim,⁹ as well as several more recent studies, evidence has accumulated to indicate that nicotinamide is methylated in the body by a transfer of methyl groups from a methyl donor. It would appear from the results of the present experiment as well as the experiment of Handler and Dubin⁴ that this transfer must be an irreversible process. In this respect N¹-methylnicotinamide would be similar to creatine.¹⁰

It has been demonstrated in several instances that a compound may be inactive as a methyl donor and yet active as a lipotropic agent or in the prevention of hemorrhagic kidneys.¹¹ With regard to the latter, we found N¹-methylnicotinamide to be inactive. In preliminary experiments which we had carried out, we had put some young rats on a purified diet containing 2% N¹-methylnicotinamide with no added choline, and these rats died in about a week from hemorrhagic kidneys. With regard to lipotropic action, Handler and Dubin⁴ found that N¹-methylnicotinamide did not prevent the development of fatty livers. On the other hand, Najjar and coworkers^{2,3} observed some lipotropic action from the compound. They pointed out³ that the slight lipotropic action which they observed might be ascribed to one of a number of factors, for example, a sparing action of the N¹-methyl-

nicotinamide on the methionine and choline in the diet which they used.

It will be noted that a small amount of choline was present in the diet during the period of feeding N¹-deuteriomethylnicotinamide in the present experiment. The choline was included in an attempt to forestall termination of the experiment by loss of the animals from severe hemorrhagic kidney involvement. If transmethylation from N¹-methylnicotinamide to choline could take place one would expect the deuteriomethyl group to appear in the isolated choline even in the presence of some choline in the diet. This assumption is based on the fact that in several experiments carried out in this laboratory, transmethylation from deuteriomethionine to choline has been found to occur even when choline was present in the diet.¹²

It is of interest that trigonelline, N-methylnicotinic acid betaine, has likewise been shown to be inactive as a methyl donor.^{8,11}

Summary. N¹-methylnicotinamide labeled in the methyl group with deuterium was fed to two rats for 6 days. Analysis of the body choline and creatine of these rats for deuterium showed that there was no detectable transmethylation from N¹-methylnicotinamide to choline or creatine during this period.

The authors wish to express their appreciation to Miss Josephine E. Tietzman for the analyses reported in this paper. The authors also wish to acknowledge a grant-in-aid from the Lederle Laboratories Division, American Cyanamid Company, which has contributed to the support of this investigation.

⁸ Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

⁹ Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

¹⁰ Simmonds, S., and du Vigneaud, V., *Proc. Soc. Exp. Biol. and Med.*, 1945, **50**, 293.

¹¹ Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **143**, 373.

¹² Schenck, J. R., Simmons, S., Cohn, M., Stevens, C. M., and du Vigneaud, V., *J. Biol. Chem.*, 1943, **149**, 355.

16245

Skeletal Changes in Growing Vitamin B Complex Depleted Rats and the Course of Repair.

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In continuation of our investigations on the response of the skeletal tissues to vitamin deficiencies, we have now analyzed the growth and development of the long bones in vitamin B complex depleted young rats. The course of repair after refeeding has also been studied.

Material and Methods. Fifty-five rats of the Wisconsin strain (24 males and 31 females), 19 to 22 days old, were divided into 3 groups, littermates and animals of the same sex being equally distributed among these groups.

Series I. 12 rats were kept on water and a stock diet composed as follows:

	g %
Yellow corn meal	51.55
Powdered whole milk	9.15
Powdered skim milk	9.15
Linseed meal	3.86
Wheat germ	1.44
Crude casein	9.63
Alfalfa leaf meal	9.63
Dried brewer's yeast	0.96
Sodium chloride	0.39
Calcium carbonate	0.39
Cod liver oil	0.96
Crisco	2.89
	<hr/> 100.00

These rats were killed after 18, 24, 30, or 40 days.

Series II. 21 animals received water and the following vitamin B-free diet:

	g %
Casein (Vit.-free)	18
Starch (Anheuser-Busch (Vit.-free)	62
Merek salt mixture No. 1	4
Cod liver oil (Mead Johnson)	2
Crisco	14
	<hr/> 100

The duration of the experiments was the same as in Series I, unless the poor condition of the animals necessitated an earlier sacrifice.

Series III. 22 rats received water and the deficient diet, but they were subsequently refeed the stock diet for 5, 10, 21 or 30 days.

As a rule, refeeding was begun after 3 weeks. The rats were weighed once a week. At the end of the experiment, the animals together with their controls were killed with chloroform. The upper end of the tibia and the lower end of the femur were removed as a whole, fixed in 10% formalin, decalcified in a formic acid-citrate mixture,¹ and embedded in paraffin. Slides, stained with hematoxylin and eosin, were prepared for histological studies. The width of the epiphyseal disc and of the cortex of the tibia was measured with an objectmicrometer, and average values were established. The number of trabeculae in the metaphysis of the tibia present in a standard area measuring 350 microns in diameter was counted.

Gross Observations. At the beginning of the observations, the mean weight of the males was 42 ± 1 g, that of the females 40 ± 6 g. During 18 days, the deficient animals, both male and female, gained 12 to 14 g, whereas the normal control males had gained 50 g and the females 68 g. Subsequently, the weights of the deficient animals dropped sharply. After 24 days, the loss was $7 \pm \frac{1}{2}$ g in males and 4 ± 3 g in females, and after 30 days, 11 ± 1 g in males and 9 ± 2 g in females. The deficient state was tolerated for 34 days by 1 male that lost 18 g. Females were more resistant, 4 animals surviving for more than 30 days, and 1 for 40 days. These females lost 16 ± 4 g. After refeeding the stock diet for 5 days, the weight of the males had increased 46 ± 7 g, that of the females 40 ± 3 g, whereas the normal controls had gained 15 g in the corresponding period of time. After 10 days, the weights of the refeed animals still remained 25 to 30% below those of the controls. After 21 days,

¹ Morse, A. J., *J. Dent. Res.*, 1945, **24**, 143.

the weights of the refed rats began to equal those of the normal ones, and after 30 days, the weights of 2 of the refed animals surpassed those of the controls.

Histologic Findings. In Males. After 18 days, the changes noted in the deficient animals were slight. The epiphyseal disc measured 320 microns (normal 355 microns). The cartilage cells were smaller and the cartilage cell rows shorter than usual. The latter were separated from each other by thin layers of chondromucoid ground substance. The single row was composed of 9 or 10 columnar and 3 or 4 hypertrophic cartilage cells (normal 12 columnar and 4 or 5 hypertrophic cells). There was mitotic proliferation of the columnar cells. The metaphysis was properly vascularized, and replacement of cartilage by bone was in progress. Whereas ordinarily the metaphyseal standard area contained 20 long osseous spicules, in the deficient animals only 16 short and thin trabeculae covered by small osteoblasts were counted. The articular



FIG. 2.

Section through the epiphyseal disc at the upper end of the tibia of a male rat, 43 days old, kept on the deficient diet for 21 days. Magnification 104 \times . Compare with Fig. 1. The epiphyseal disc is narrowed and bone formation is markedly decreased.

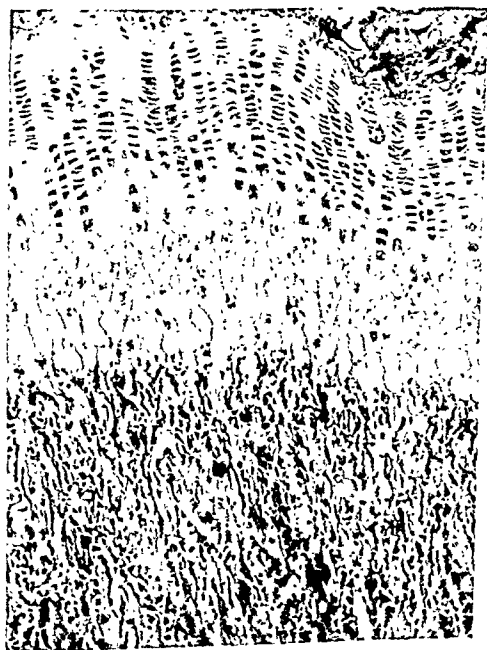


FIG. 1.

Section through the epiphyseal disc at the upper end of the tibia of a male rat, 44 days old, receiving the stock diet for 25 days. Magnification 104 \times .

cartilage cells were small. The cortex was 225 microns thick (normal 255 microns). The endosteum contained a continuous layer of oblong osteoblasts. The vascular canals were enlarged; the periosteum was dense; the bone marrow was hemopoietic.

After 24 days, the epiphyseal discs of the deficient animals had narrowed down to 135 microns (Fig. 1, 2). The shortened cell rows were composed of 6 to 8 small columnar and 2 or 3 small sclerosed "hypertrophic" cartilage cells. Mitoses were not seen. The cartilaginous matrix was abundant and hyalinized. The vascularization of the metaphysis was diminished, and only 5 trabeculae were found in the standard area. The spicules were very short, and frequently only fragments of bone were seen. The cartilage cells of the pressure and hypertrophic zones of the joint were less numerous and smaller than ordinarily. The cortex measured 205 microns. The endosteum consisted of a discontinuous layer of small

osteoblasts or spindle cells with elongated cytoplasm. The vascular canals were filled with edematous connective tissue. The periosteum was loosened. In the bone marrow, foci of serous atrophy were noted. Subsequently, the changes were still more pronounced.

After 30 days, growth of cartilage and bone was at a standstill. The single cartilage cell row contained 5 or 6 columnar and 2 small hypertrophic cells. Much hyaline matrix appeared between the cartilage cell rows, and a layer of preosseous material was seen in the distal third of the atrophic epiphyseal disc. Metaphyseal trabeculae were absent. Instead, a thin transverse bony lamella delimited the epiphyseal cartilage from the marrow cavity. The cartilaginous covering of the joint was markedly atrophic. The cortex of the shaft measured 135 microns. At its inner surface, a discontinuous layer of small spindle cells had taken the place of osteoblasts. The Haversian canals were enlarged and contained engorged vessels and edematous connective tissue. The periosteum was poor in cells. The bone marrow showed an advanced state of serous atrophy.

After 34 days, the epiphyseal disc was only 65 microns wide, and the single cartilage cell row was composed of 4 inactive columnar cells and 1 cell of hypertrophic type. The conditions were otherwise comparable to those seen after 30 days.

In Females. The changes were of the same kind as in the males, but their progress was slower. Thus after 18 days on the deficient diet, the epiphyseal disc was 330 microns wide (normal 340 microns), and the single cartilage cell row contained 10 or 11 columnar and 4 hypertrophic cells. The decrease in the size of the cartilage cells, however, was conspicuous. There were 18 trabeculae in the metaphyseal standard area (normal 20). The cortex was of usual diameter (245 microns). The endosteum and periosteum were unchanged.

After 24 days, the epiphyseal disc was 165 microns high. The cartilage cell rows were short and composed of 7 to 9 columnar and 4 hypertrophic cells. Both cell types were considerably smaller than usual. Hyaliniza-

tion of the cartilaginous matrix was less accentuated than in the male. In the metaphysis, 8 short trabeculae covered by small osteoblasts were counted. The cortex was 225 microns thick. The changes in the endosteum and periosteum were now comparable to those seen in the male. In the bone marrow, a few foci of serous atrophy were noticeable.

After 30 days, conditions in the female had approached those in the male. After 40 days, the epiphyseal disc had narrowed down to 70 microns, the single cartilage cell row containing 3 atrophic columnar cells and 1 hyalinized "hypertrophic" cell. Trabeculae were lacking. The articular cartilage cells were markedly atrophic. The cortex of the shaft was 145 microns thick. The bone marrow had undergone complete serous atrophy.

Effects of Refeeding. After 5 days, the growth of the epiphyseal and articular cartilages as well as endochondral and periosteal

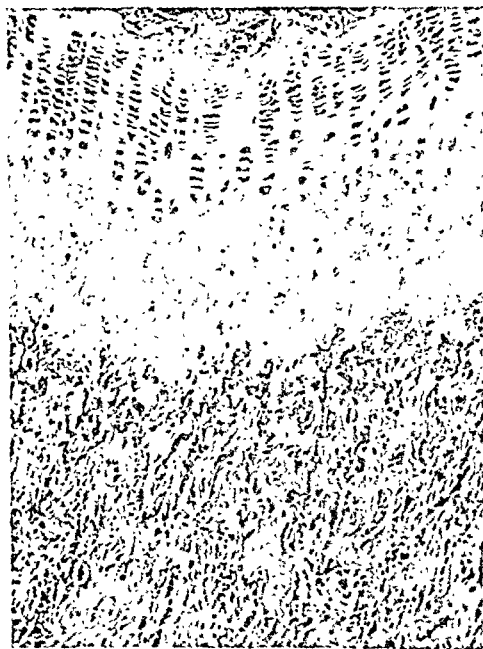


FIG. 3.

Section through the epiphyseal disc at the upper end of the tibia of a male rat, 45 days old, kept on the deficient diet for 21 days, and refed the stock diet for 5 days. Magnification 104 \times . Compare with Fig. 2. Growth of the epiphyseal cartilage and endochondral ossification are resumed.

ossification was resumed with remarkable intensity. Mitotic proliferation of the columnar cartilage cells was accentuated, and their conversion into hypertrophic cells was intensified. In the male, the epiphyseal disc was 355 microns high (Fig. 3). There was little intercartilaginous ground substance present. The single cartilage cell row was composed of 12 or 13 columnar and 4 or 5 hypertrophic cells. The standard area of the metaphysis contained 18 thick and long spicules covered by large mitotically proliferating osteoblasts. The cortex measured 235 microns. The endosteum was again composed of large osteoblasts, and the bone marrow was completely regenerated. After 10 days, the epiphyseal disc was 400 microns high. The single cartilage cell row contained 13 or 14 columnar and 5 large hypertrophic cells. There were 27 thick interlacing trabeculae in the standard area. The cortex was 265 microns thick. Endosteum and periosteum were not remarkable. The intensification of ossification was even more pronounced in the female than in the male. Gradually, the growth processes declined and assumed a normal rate in both sexes. After 21 or 30 days of refeeding, the histological appearance of the skeletal tissues did not differ from that of the normal controls of corresponding sex and age.

Comment. In vitamin B complex deficiency, skeletal development continues for some time, although at a reduced rate. The vitamin reserves of the organism are apparently sufficient to permit these processes to go on for a time. The subsequent cessation of growth and the serous atrophy of the bone marrow are findings typical of inanition. They suggest that the food ingested was not utilized, probably because the vitamin reserves had become exhausted. The severity of these changes manifests itself when they are compared with those obtained previously in underfed growing guinea pigs² and rats.³ In animals kept on a quantitatively restricted but adequately balanced diet, skeletal growth pro-

ceeded at a reduced rate, but it lasted longer than usual. Thus in rats allowed to gain only 5 g every 50 days throughout their life, the long bones were only 13 to 18 % shorter than in normally fed animals.³ In other words not unless the utilizable food energy is at a minimum and probably incompatible with life, can the inherent growth tendency of the skeleton be completely suppressed. The lack of thiamine in the diet may be responsible for loss of appetite and consequently decreased intake of food. However, the advanced skeletal changes seen in vitamin B complex-depleted rats were out of proportion to the diminished intake of food and must thus be attributed to the lack of the vitamin B complex. Again, the male responded to the vitamin deficiency more readily than the female. At this early age, the growth rate of the male is higher than that of the female. Any factor injurious to growth must, under otherwise identical conditions, necessarily affect the former more severely than the latter. If refeeding was begun before the animals were moribund, skeletal growth was quickly resumed, surpassed temporarily the normal rate and finally returned to normal. These findings are in agreement with observations in the skeleton of undernourished and refed animals.²⁻⁵

The present findings in vitamin B complex-depleted rats were similar to but more marked than those seen in mice deficient in pantothenic acid, or pyridoxine.⁶ Lack of riboflavin, on the other hand, inhibited growth less but produced more degenerative changes in the epiphyseal cartilage than total B complex deficiency.⁷ Serous atrophy of the bone marrow was not observed if only one of the aforementioned components of the vitamin B complex was lacking in the diet.

Investigations are in progress to determine

⁴ Jackson, C. M., *The Effects of Inanition and Malnutrition upon Growth and Structure*, P. Blakiston's Son & Co., Philadelphia, 1925.

⁵ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, **18**, 95; 1915, **23**, 439.

⁶ Silberberg, R., and Levy, B. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, in press.

⁷ Levy, B. M., and Silberberg, R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 355.

² Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, **30**, 675.

³ Saxton, J. A., Jr., and Silberberg, M., *Am. J. Anat.*, 1947, **81**, 455.

whether the changes seen in complete vitamin B complex deficiency represent a cumulative effect of all the constituents of the complex; or whether in the absence of one of the constituents the others, even if present in the diet, cannot be properly utilized. The latter condition might then be comparable to that shown to exist relative to the essential amino acids.⁸

Summary. In growing vitamin B complex-deficient rats, there is retardation and, finally,

cessation of growth of cartilage and bone associated with serous atrophy of the bone marrow. The male is more sensitive to the deficiency than the female. After refeeding a stock diet, skeletal growth is resumed at an intensified rate resulting in early restoration of normal conditions.

⁸ Cannon, P. R., Steffee, C. H., Frazier, L. J., Rowley, D. A., and Stepto, R. C., *Fed. Proc.*, 1947, 6, 390.

16246 P

Toxicity of Equine Serum Treated by Alkali.

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Observations on the removal of antigenicity of proteins by alkali were early made by Wells,¹ TenBroeck² and have been confirmed and extended by a number of later workers. Recently, Davis and Eaton,³ as well as Kazal, DeFalco and Arrow,⁴ paid special attention to the toxicity of the resulting products but came to apparently conflicting conclusions. The former found alkali-treated bovine serum albumin to be non-toxic for white mice, rabbits, and for 2 dogs when given intravenously. On the other hand, Kazal *et al.* confirmed an earlier report by Lewis⁵ that purified bovine plasma protein treated by sodium hydroxide gained acute toxicity for guinea pigs as soon as its antigenicity was lost. In view of the importance of these findings especially in relation to possible practical application, it seems worthwhile to make a similar study on horse serum treated by alkali and to observe its toxicity in rabbits, guinea pigs, and white mice. The results obtained are herewith presented.

After several preliminary trials, it was found that one-quarter normal solution of sodium hydroxide would, after a period of 30 days at 37°C, render normal horse serum so altered in antigenicity that rabbits immunized with the treated serum produced no precipitin against both normal and treated sera, gave occasional complement fixation reaction with either, and in half of these immunized animals, skin allergy to normal serum alone was demonstrated. Furthermore, of 10 guinea pigs sensitized to treated serum, only 3 out of 7 reacted mildly with normal, and 4 out of 6 similarly to alkali-treated serum without a single death. At the same time, 4 controls died when shocked with normal serum. Nine rabbits tolerated as much as one gram of treated serum protein per kilo body weight without any symptoms, whereas only 5 out of 12 immunized rabbits reacted with an elevation of body temperature to half a degree Centigrade following intravenous injections of 20 ml (800 mg protein) of the treated serum. Among 26 white mice weighing 20 g given intravenously 0.5 ml of the same (20 mg protein), 2 died in 6 hours, and one died in a week. The rest survived 4 weeks, the period of observation. However, among 9 guinea pigs weighing 200-250 g, only 2 out of 3 receiving less than 100 mg of treated serum protein intracardially survived, while all 6

¹ Wells, H. G., *J. Inf. Dis.*, 1909, 6, 506.

² TenBroeck, C., *J. Biol. Chem.*, 1914, 17, 369.

³ Davis, H. A., and Eaton, A. G., *Proc. Soc. Exp. Biol. and Med.*, 1942, 59, 246.

⁴ Kazal, L. A., DeFalco, R. J., and Arrow, L. E., *J. Immunol.*, 1946, 54, 245.

⁵ Lewis, J. H., *Science*, 1943, 98, 371 (quoted in 4).

receiving 120-240 mg of the same died almost immediately, following intracardial injection, from shock symptoms not unlike those described by Kazal *et al.*

Thus it seems clear that, as in the case of alkali-treated bovine serum, alkali treatment of equine serum rendered it practically non-

antigenic, but definitely toxic for guinea pigs. At the same time, it remained practically innocuous for rabbits and for white mice. The toxicity of treated serum for larger laboratory animals and the nature of this toxic substance will be the subjects of further investigation.

16247 P

Treatment of Euthyroid Cardiac Patients by Producing Myxedema with Radioactive Iodine.*

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Significant clinical improvement in euthyroid cardiac patients following total thyroidectomy has been reported.¹ The rationale of this procedure was verified in that the degree of alleviation of angina pectoris and congestive failure was generally proportional to the degree of reduction in the metabolic rate.² The advent of radioactive iodine has raised the possibility of achieving the same result.

Abstract of Cases. *Case 1.* EM, 46-year-old woman, entered the hospital in 1941 because of progressive ankle edema, dyspnea and orthopnea during the previous year. She had chorea at the age of ten. Physical examination revealed rheumatic heart disease, cardiac enlargement, mitral and aortic stenosis and insufficiency, auricular fibrillation, congestion of the lungs and liver, ankle edema

and orthopnea. She improved after one week in the hospital. In 1942, symptoms and signs of thyrotoxicosis appeared; the basal metabolic rate was approximately plus 33%. A maximal subtotal thyroidectomy was done in 1943. Myxedema developed following this procedure; the signs and symptoms of congestive heart failure markedly improved and she returned to work. Thyroid extract, 0.065 g, was administered daily until January, 1947, when, for the fifth time, the patient entered the hospital with orthopnea, pleural effusion, ascites, and peripheral edema. Thyroid medication was omitted. Despite adequate digitalization, mercurial diuretics, and salt and water restriction, little improvement resulted during 3 weeks of treatment. Following discharge from the hospital, the above signs and symptoms became progressively worse, paroxysmal nocturnal dyspnea appeared, and she again entered the hospital on June 14, 1947. Slight improvement resulted from the above regime of treatment. The basal metabolic rate averaged approximately plus 5%, the serum cholesterol was 220 mg/100 cc. On July, 5, '47, 5 millicuries I^{131} were administered. The total 3-day urinary excretion was 71%. Approximately 10 weeks later the basal metabolic rate was minus 14% and the serum cholesterol 400 mg %. On November 1, '47, 13.6 millicuries I^{131} were administered. A tracer study on December 4, 1947, showed

* This work was carried out under a contract between the Office of Naval Research and the President and Fellows of Harvard College.

† The authors wish to acknowledge the helpful advice of Dr. Arthur K. Solomon of the Department of Biophysics, Harvard Medical School, and of Mr. Leonidas Marinelli of the Memorial Hospital, New York City.

¹ Blumgart, H. L., Levine, S. A., and Berlin, D. D., *Arch. Int. Med.*, 1933, **51**, 866.

² a. Blumgart, H. L., Berlin, D. D., Davis, D., Riseman, J. E. F., and Weinstein, A. A., *J. A. M. A.*, 1935, **104**, 17; b. Blumgart, H. L., Riseman, J. E. F., Davis, D., and Weinstein, A. A., *Am. Heart J.*, 1935, **10**, 596.

84% excretion in 3 days. On December 10, '47, 6 weeks after the second dose, the basal metabolic rate was minus 26% and the serum cholesterol 440 mg %. Clinical evidence of myxedema, including lethargy, hoarseness, dry hacking cough, cold dry yellow skin were present. Her cardiac status improved. She was able to sleep flat in bed and undertake considerably more effort without dyspnea. Peripheral edema reaccumulate more slowly and, therefore, mercurial diuretics were required less frequently. Thyroid extract, 6 mg per day, mitigated the symptoms of myxedema and has enabled the patient to maintain clinical improvement.

Case 2. 60-year-old man entered the hospital for the fifth time on June, 1947, because of progressive cyanosis, dyspnea, orthopnea, hydrothorax, and nightly attacks of acute paroxysmal dyspnea. He had been treated in the out-patient department and wards for more than 5 years. The clinical diagnoses were arterial hypertension, rheumatic heart disease, mitral and aortic stenosis and insufficiency, marked cardiac enlargement, auricular fibrillation, arteriosclerosis of the coronary and peripheral arteries, and congestive heart failure. Despite treatment including optimal digitalization, mercurial diuretics, restricted fluid and salt intake, and curtailment of activity, his clinical status became progressively worse. Following admission to the hospital, treatment, including the removal of 1700 cc from the right chest which had accumulated in one week since previous thoracentesis, resulted in slight improvement, but he continued to have paroxysmal dyspnea and was considered critically ill. Basal metabolic rates ranged from plus 8 to minus 8% and the serum cholesterol level was 200 mg %. Ten millicuries I^{131} were administered on July 5, 1947. The total 3-day urinary excretion was 51%. On September 18, 1947, an additional 2.25 millicuries of I^{131} were administered. Four weeks later, or $3\frac{1}{2}$ months following the first dose, the basal metabolic rate had declined to minus 25% and the serum cholesterol increased to

600 mg %. Coincidentally, the clinical status of this patient improved. He was able to sleep uninterruptedly flat in bed and dyspnea was much less, despite increased activity up and about on the ward. Reaccumulation of fluid has been slower and he requires fewer injections of mercurial diuretics.

Discussion. In both patients, the uptakes of tracer doses of I^{131} were consistent with results obtained in subjects with normal thyroid glands. The normal basal metabolic rates, the normal serum cholesterol values, the normal cardiac rates and the absence of any clinical evidences of thyrotoxicosis were consistent with the normal thyroid state. The elevation of the serum cholesterol values in reciprocal relation to the fall in the basal metabolic rates which occurred gradually as the clinical evidences of myxedema appeared, demonstrate that radioactive iodine produced hypothyroidism in these two patients. The clinical improvement concomitant with reduction in metabolic rate observed in these two patients was similar to that observed in previous studies of cardiac patients following surgical total thyroidectomy.²

Considerable attention was given to possibly toxic effects of the rather large doses of radioiodine administered to these patients. Our studies have failed to disclose such evidence. The optimum dosage and regime of administration of I^{131} , the regularity with which myxedema may be induced in such patients, the possible utilization of adjuncts, such as thio-urea derivatives and thyroid stimulating hormone of the pituitary, and final appraisal of the clinical value of this procedure, are matters under investigation which prolonged and extensive studies and observations will clarify.

Summary. Radioactive iodine 131 was administered to 2 severely ill cardiac patients who showed no clinical or laboratory evidences of thyrotoxicosis or myxedema. In both instances, decided reduction in the metabolic rate coincided with clinical improvement. These results justify further studies and observations.

Insulin Hypoglycemia in Chick Embryos.*

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Landauer^{1,2} has found that certain morphological abnormalities may be produced by injecting insulin into the yolk of 0-5-day chick embryos. In the course of an investigation into the carbohydrate metabolism of insulin-treated embryos we have found that a persistent hypoglycemia is produced by insulin applied in this manner. This phenomenon is interesting because the hormones usually related to insulin activity are probably not present during the first 7-8 days of embryonic life.

The only certain information about the pituitary gland concerns the onset of cytological differentiation. Rahn³ has shown that acidophiles and basophiles do not appear before the 10th day (though both are preceded by a general basophilic tendency starting on the 8th day). The adrenal cortex (Okuda⁴) is not formed until the 6th day; when cortin first appears is uncertain. Islets of Langerhans are not differentiated until 8-9 days and detectable amounts of insulin do not appear until the 11th day. Needham⁵ has emphasized that there is no significant glycolytic system in embryonic chick tissues during the earlier stages.

Two units of insulin (Iletin, Lillie) were injected into the yolk sac of 5-day embryos.

Blood was obtained directly from the extra embryonic blood system by means of suction through micro cannulae inserted into the vessels. Different groups of embryos were bled on successive days following the injection. Analyses for sugar were made on .01 ml samples with a modified Folin-Malmros technic. The average error for 45 samples of a standard (100 mg % glucose) solution with this procedure was 5.4 mg; standard error was 8.8 mg. The data are presented in Table I. It can be seen that the hypoglycemia is pronounced 24 hours after the injection and that there is a recovery from the insulin effect some time between the 12th and 14th days (7-9 days after injection). Various authors have claimed that normally the embryo's insulin becomes an important factor in control of blood sugar level at this time (Needham⁶).

There is some evidence that a hypoglycemia may be produced at still earlier stages. Insulin (2 units) was injected into the yolk sac of 30-hour embryos. Blood samples were taken 2 days later. At this stage it was necessary to pool blood from two embryos to obtain a .01 mg sample. The average blood sugar value for 4 such samples was 106.9 mg % while that for controls was 145.7 mg %.

TABLE I.
Average of Blood Sugar Values in mg %: 2 units of insulin injected at 5 days.
(No. of cases in parentheses.)

Days of incubation	6	7	8	9	10	12	14	16
Uninjected controls	135 (11)	145 (8)	145 (8)	147 (6)	134 (9)	146 (6)	160 (10)	157 (5)
Insulin treated	80 (16)	70 (10)	76 (8)	49 (6)	58 (8)	53 (5)	135 (8)	141 (3)

* This work was supported, in part, by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

¹ Landauer, W., *J. Exp. Zool.*, 1945, **98**, 65.

² Landauer, W., *J. Exp. Zool.*, 1947, **105**, 145.

³ Rahn, H. J., *Morph.*, 1939, **64**, 483.

⁴ Okuda, M., *Endocrinol.*, 1928, **12**, 342.

⁵ Needham, J., *Biochemistry and Morphogenesis*, 1942.

⁶ Needham, J., *Chemical Embryology*, 1931.

Total Serum Protein Concentration and Total Weight of Circulating Protein in the Rat.

RICHARD W. LIPPMAN.* (Introduced by T. Addis.)

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The relation of total serum protein concentration and total weight of circulating protein to body size and sex were studied in the rat. The animals were used concurrently for estimation of blood and plasma volumes reported previously.¹

Methods. A total of 125 normal rats were used, 47 females and 78 males, ranging in weight from 48 to 308 g. The rats were divided into groups, each of approximately 10 animals. In each group, the weight range was less than 10% of the mean weight, in most cases considerably less.

Blood and plasma volumes were determined by the hemoglobin dye method previously described.¹ Serum from the rats in each group was pooled in aliquot portions, and the total serum protein concentration was measured by a modification of Kingsley's biuret method.²

Because hemoglobin was used in determining plasma volumes, it was necessary to correct the protein determinations. This was done by subtracting from the total circulating protein value the weight of hemoglobin injected, and by subtracting from the total serum protein concentration the calculated concentration of hemoglobin. The weight of hemoglobin injected was determined by the

colorimetric method of Evelyn and Malloy.³

In this paper, the previously reported individual values for blood and plasma volumes of the rats within each group were averaged. The mean values of these groups were related to body surface area, calculated from the formula of Carman and Mitchell.⁴

Results. The blood and plasma volumes (Fig. 1), total serum protein concentration (Fig. 2), and total circulating serum protein (Fig. 3) were plotted against body surface area. The results fitted a straight line reasonably well in each category. Regression lines

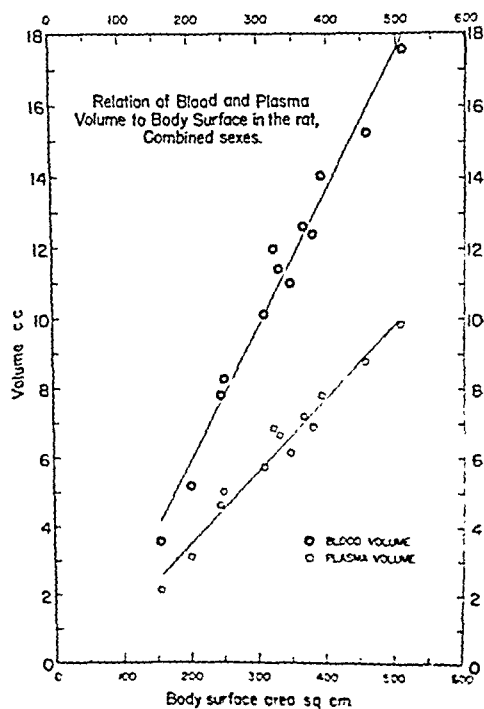


FIG. 1.

* The author wishes to acknowledge gratefully the technical assistance of Helen J. Green. This work was aided by a grant from the U. S. Public Health Service. Hemoglobin solution was provided by Sharp & Dohme, Inc., Philadelphia, Pa. Dr. Lippman is now at the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

¹ Lippman, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 188.

² Kingsley, G. R., *J. Biol. Chem.*, 1939, 131, 197.

³ Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, 126, 655.

⁴ Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, 70, 350.

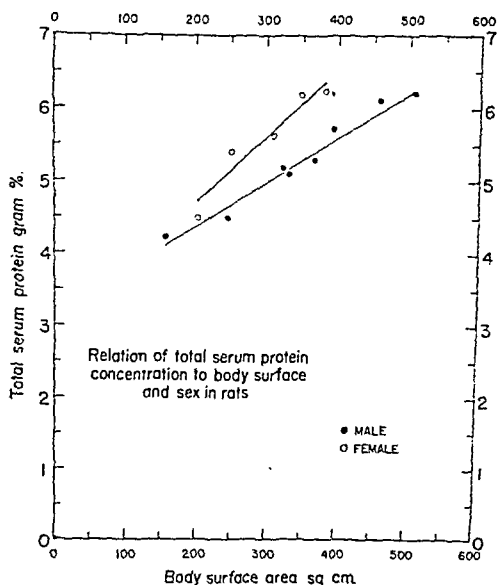


FIG. 2.

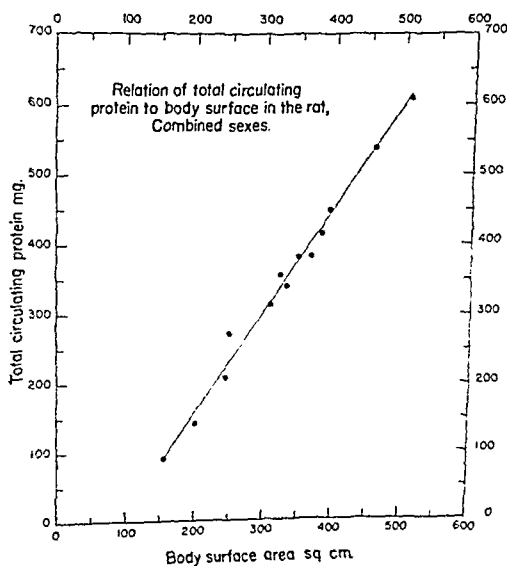


FIG. 3.

were calculated and the constants are given in Table I. There were slight sex differences in the regression constants for blood and plasma volumes and for total circulating protein, but as these did not appear significant for the purposes of this paper, the data were combined and are given in a single regression line. A significant sex difference in total serum protein concentration was found, and 2 re-

gression lines are presented (Fig. 2).

Discussion. Few previous studies of the total serum protein concentration or the weight of total circulating serum protein in the rat have been made. Comparison is difficult, as different methods have been used to express results.

Cutting and Cutter⁵ determined circulating plasma protein and plasma protein concentrations in rats. Their data were not segregated by sex, and they divided the rats into only 2 groups: "large" (292-395 g) and "small" (188-213 g). Their results, given in Table II, demonstrate a difference in the plasma protein concentration between the 2 groups. In addition, they found that the total circulating plasma protein in the large adult rats was greater in terms of surface area than in the smaller adult rats. Because female rats of the colony used by Cutting and Cutter rarely grow to weights over 300 g, it may be assumed that their "large" group consisted of male rats.

Metcoff and Favour⁶ determined the total plasma protein concentration and total circulating plasma protein of rats, and likewise did not segregate their data by sex. They divided the rats into 3 groups: adult (119-335 g), pubescent (73-89 g), and prepubescent (40-68 g). Their total plasma protein concentrations were somewhat higher than other values reported, and they attributed this difference to the copper sulfate method of determination, which they used. However, their range of difference in total plasma protein concentration, between small rats and large rats, is similar to that which we found. They also found that the total circulating plasma protein increases in terms of body surface up to puberty, after which it remains relatively constant.

Our data, segregated by sex and into narrow weight ranges, appear to demonstrate a constant relationship of total circulating serum protein and serum protein concentration to body surface area over a wide range of weight which includes the range of puberty.

⁵ Cutting, W. C., and Cutter, R. D., *Am. J. Physiol.*, 1935, **113**, 150.

⁶ Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **142**, 94.

TABLE I.

Relation of Blood Volume, Plasma Volume, Total Circulating Serum Protein, and Total Serum Protein Concentration to Body Surface Area.

General equation: $f(x) = A + Bx$

where x is body surface area in sq cm.

$f(x)$	Sex	A	B
Blood volume (cc)	♂	— 1.59	0.0385 ± .0032
	♀	— 1.90	0.0380 ± .0048
	Combined	— 1.91	0.0390 ± .0016
Plasma volume (cc)	♂	— 0.570	0.0211 ± .0026
	♀	— 0.316	0.0192 ± .0026
	Combined	— 0.710	0.0211 ± .0008
Total circ. protein (mg)	♂	—150.	1.50 ± .04
	♀	—147.	1.54 ± .06
	Combined	—142.	1.49 ± .04
Serum protein conc. (g %)	♂	3.15	0.00617 ± .0024
	♀	2.85	0.00920 ± .0026

TABLE II.

Previously Reported Values for Plasma Protein Concentration and Weight of Total Circulating Plasma Protein.

Author	Wt group	Total plasma protein conc. g %	Total circ. plasma prot. mg/100 sq cm
Cutting and Cutter	Large (292-395 g)	6.88	108
	Small (188-213 g)	6.30	82
Metcoff and Favour	Adult (119-335 g)	8.07	190
	Pubescent (73-89 g)	6.60	172
	Prepubescent (40-68 g)	5.62	118

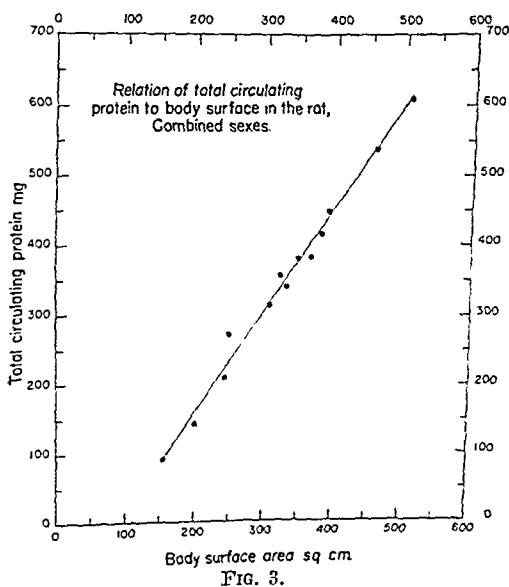
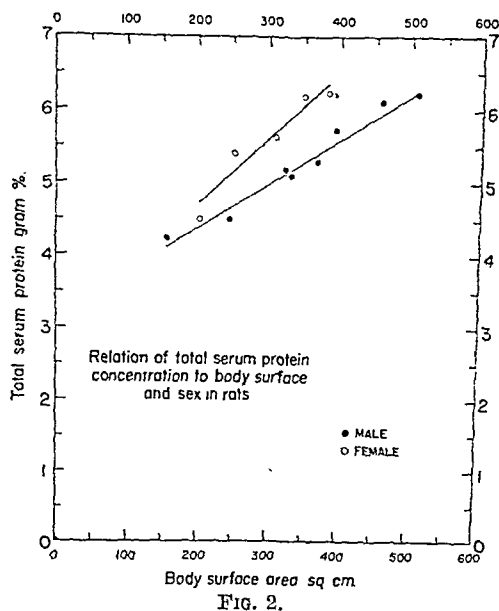
This apparent discrepancy between our observations and those previously reported may be explained by the fact that the regression lines do not intercept the x -axis at 0, so that the ratio $f(x)/x$ increases as x increases. Although the lines are valid over the observed range, the relationship must change in extremely young rats, as it must ultimately pass through the point of origin.

We have related our results to body surface area of the rat, although we realize the imperfections of body surface as a reference measure. Kleiber⁷ has clearly discussed the

errors of body surface area calculations and relates metabolic rate to the 0.75 power of body weight. Our blood and plasma volume data exhibit a closely similar relationship.¹ We have used the relation of variables to body surface area here because it affords some comparison with other discussions in the literature.

Summary. 1. The total circulating serum protein and the serum concentration of total protein in the rat are shown to be related to body surface area over a wide range. 2. The sex difference in total serum protein concentration is of sufficient magnitude to require its consideration in experiments.

⁷ Kleiber, M., *Physiol. Rev.*, 1947, **27**, 511.



were calculated and the constants are given in Table I. There were slight sex differences in the regression constants for blood and plasma volumes and for total circulating protein, but as these did not appear significant for the purposes of this paper, the data were combined and are given in a single regression line. A significant sex difference in total serum protein concentration was found, and 2 re-

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Metcoff and Favour⁶ determined the total plasma protein concentration and total circulating plasma protein of rats, and likewise did not segregate their data by sex. They divided the rats into 3 groups: adult (119-335 g), pubescent (73-89 g), and prepubescent (40-68 g). Their total plasma protein concentrations were somewhat higher than other values reported, and they attributed this difference to the copper sulfate method of determination, which they used. However, their range of difference in total plasma protein concentration, between small rats and large rats, is similar to that which we found. They also found that the total circulating plasma protein increases in terms of body surface up to puberty, after which it remains relatively constant.

Our data, segregated by sex and into narrow weight ranges, appear to demonstrate a constant relationship of total circulating serum protein and serum protein concentration to body surface area over a wide range of weight which includes the range of puberty.

⁵ Cutting, W. C., and Cutter, R. D., *Am. J. Physiol.*, 1935, **113**, 150.

⁶ Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **142**, 94.

TABLE I.
Percentage Composition of Diets.

Constituent	Stock diet (17% prot.)	3% protein	77% protein	10% dext.	10% dext. with salt
Bone ash	1.5				
Soy bean meal	5.7				
Wheat	73.4				
Corn starch	4.0	74.0			
Casein	9.7		74.0		
Yeast		10.0	10.0		
Sardine oil	3.2	10.0	10.0		
Alfalfa	2.0	2.0	2.0		
Salt mixture	0.5	4.0	4.0		
NaCl					0.4
Vit. B complex				1.0	1.0
Dextrose				10.0	10.0
Water				89.0	88.6

TABLE II.
Effect of Various Regimens on Blood and Plasma Volume and Serum Protein.
Mean Results.

Diet	Total starv.	10% dext.	10% dext.	10% dext.	10% dext.	3% prot.	77% prot.
Duration	17 hr	17 hr	17 hr	10% dext.	10% dext.	7 days	7 days
No. rats	9	16	20	8	8	20	20
Initial wt, g				151	153	172	172
Final wt, g	147	173	163	119	125	155	185
Blood vol., cc	11.29	11.91	12.56	9.67	10.01	11.57	13.68
% normal	109 ± 1.4	99 ± 2.1	112 ± 1.1	110 ± 2.3	110 ± 2.3	107 ± 2.6	110 ± 2.3
Plasma vol., cc	6.22	6.66	7.14	4.90	5.35	6.10	7.52
% normal	103 ± 1.8	100 ± 2.6	111 ± 1.4	96 ± 3.4	103 ± 3.8	99 ± 2.5	106 ± 2.3
Total serum protein, g %	5.33	5.42	5.28	4.28	4.48	4.70	5.62
% normal	105	102	101	89	92	92	102
Total circ. protein, mg	332	361	377	210	240	287	423
% normal	102	96	106	81	87	84	106

after 9 to 11 days on a protein-free diet. Frisch, Mendel and Peters⁵ produced marked depression of serum protein concentrations by using a carrot diet for 18 weeks. Bloomfield,⁶ on the contrary, found that an otherwise balanced 3% protein diet did not produce much lowering of serum protein concentration at the end of 21 weeks.

Metcoff, Favour and Stare⁷ studied protein deficiencies in the rat, and their conditions for "acute protein deficiency" closely resem-

bled our 3% protein diet for 7 days. They found, under these conditions, a decrease in the circulating total protein per unit surface area, and in the plasma volume per unit surface area.

The apparent increase that we found in the cell volume of rats with protein deficiency is probably due to the stability of the erythrocyte mass compared with the lability of plasma volume and body weight. In one week, the erythrocyte mass formed at the starting weight would not have changed appreciably, while the plasma volume and body weight had diminished, so that the erythrocyte mass was relatively increased.

We have avoided using the relation of variables to body surface area in these comparisons because of the pitfalls that have

⁴ Boycott, A. E., and Chisolm, R. A., *J. Path. and Bact.*, 1911, **16**, 263.

⁵ Frisch, R. A., Mendel, L. B., and Peters, J. P., *J. Biol. Chem.*, 1929, **84**, 167.

⁶ Bloomfield, A. L., *J. Exp. Med.*, 1933, **57**, 705.

⁷ Metcoff, J., Favour, C. B., and Stare, F. J., *J. Clin. Invest.*, 1945, **24**, 82.

Effects of Protein and Fluid Consumption Upon Plasma Volume and Circulating Protein in the Rat.

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In studying rats under experimental conditions, changes in the concentration of serum constituents were noted. It was considered possible that such changes might be the consequence of changes in blood and plasma volume, induced by alterations in the consumption of food and fluid, rather than the consequence of a direct metabolic effect. This study was undertaken in order to determine whether the conditions of our experiments had produced such alterations in blood and plasma volumes.

Methods. A total of 101 normal male rats were used, ranging in weight from 148 to 196 g. The rats were taken from stock, where they had received a diet containing 17% of protein, and water as desired. They were placed in individual cages and given experimental diets for a specified time. At the end of this time, blood and plasma volume determinations were made by the method previously reported.¹ The serum from each experimental group of 8 to 10 rats was pooled in aliquot portions, and the total serum protein was determined by a modification of Kingsley's biuret method.² Exact composition of the diets is given in Table I. Total circulating protein was calculated from the measured data.

The rats received unrestricted amounts of the 3% and 77% protein diets and water. With the exception of the total starvation

group, each group received an adequate number of calories.

Results. Detailed results are given in Table II, and compared with normal control values previously established for rats of the same weight taken directly from stock diet.³

None of the protein-deficient diets, whether of high or low fluid content, induced any appreciable change in plasma volume at the end of 7 days, although the total blood volume was increased about 10% when compared with normal for the final weight.

The fluid diet with added salt appeared to increase both total blood volume and plasma volume about 10% at the end of 17 hours. The 77% protein diet produced a similar change at the end of 7 days.

Neither the fluid diet without salt, nor total starvation for 17 hours altered the total blood or plasma volume.

None of the 17-hour experiments showed any significant change in total circulating serum protein or total serum protein concentration. However, all the protein-deficient diets reduced the total circulating serum protein by 15-20% in 7 days, with an almost commensurate reduction in the total serum protein concentration. The 77% protein diet did not produce any significant change in total circulating serum protein or in the total serum protein concentration during the period studied.

Discussion. Although there have been many studies of the effect of diet upon total serum protein concentration, very few have correlated such effects with changes in the blood and plasma volumes, or have studied volume changes alone.

Boycott and Chisolm⁴ found no change in blood volume, but a fall in plasma volume,

* The author gratefully acknowledges the technical assistance of Helen J. Ureen and Way Lew. This work was aided by a grant from the U. S. Public Health Service. Hemoglobin solution was provided by Sharp and Dohme, Inc., Philadelphia, Pa. Dr. Lippman is now at the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

¹ Lippman, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 188.

² Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

³ Lippman, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 193.

TABLE I.
Effect on Body Weight, Food Intake, and Liver Fat of Adding Methionine and Choline to a Diet Free of Sulfur-Containing Amino Acids.

Group	Special supplement (daily)	No. of rats	Weight of animals		Food intake, g/day	Liver	
			Initial, g	Change, %		Wt, g	Total lipid, %
I	None	12	156 (150-164)	-32 ± 1.4	4.2 ± 0.18	4.7 ± 0.12	20.4 ± 1.6
II	50 mg Methionine	12	155 (150-165)	-26 ± 1.2	4.1 ± 0.13	4.3 ± 0.15	13.3 ± 1.3
III	100 " "	12	155 (150-160)	-27 ± 1.9	4.1 ± 0.17	4.2 ± 0.15	9.7 ± 0.75
IV	25 " Choline	12	154 (150-162)	-29 ± 1.1	4.0 ± 0.11	3.8 ± 0.12	6.8 ± 0.36

significant reduction of liver fat at the 50 mg level (average fat, 13.3%), with still lower values when 100 mg were given (average fat, 9.7%). This result, with the lower food intake and greater weight loss in this experiment, suggests that the 50 mg level of methionine was at the borderline of effective dosage, and in the present case, the higher ratio of methionine in the diet (1.2%) was sufficient to produce a lipotropic effect.

When choline was given it showed its usual efficient lipotropic action. The average liver fat content of the choline-fed group was 6.8%, an almost normal value. Methionine in the 100 mg dosage gave a 40% higher fat content of

the liver. The total methyl in this amount of methionine is 25% greater than in the choline.

It may be noted that the weight of the livers paralleled the fat content consistently.

Summary. Rats fed an alipotropic diet in which a mixture of amino acids replaced protein developed fatty livers in spite of low food intake and emaciation. Methionine in adequate dosage exerted a lipotropic effect although there was still 10% of fat in the liver when the methionine constituted 2.4% of the diet. When 25 mg of choline chloride per day were fed the livers were essentially normal.

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Therapeutic Effectiveness of Single and Divided Doses of Penicillin in a Streptococcal Infection in Mice.

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In a preliminary study on penicillin dosage and its therapeutic effectiveness it was found that a single oral dose containing 10 mg of penicillin G per kg of body weight protected a large proportion of mice in an otherwise fatal experimental streptococcal infection.¹ The results of further studies on the effect of single doses of drug, administered orally or

subcutaneously, are given in the present communication.

In addition, a comparison between the effect of a given amount of penicillin when administered as a single dose and as a series of equally spaced smaller doses has been included in this report. Such a comparison is of interest, since it is commonly assumed that the therapeutic effectiveness of penicillin in aqueous solution depends upon repeated

¹ White, H. J., Lee, M. E., and Alverson, C., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 35.

been suggested previously.³ The situation would be additionally complicated by the fact that body surface formulae are derived from rats on stock diet. It is known that, after starvation or over-feeding, the composition of the body changes with respect to the basic constituents such as protein.⁸ With such changes, the relation between body weight and surface area may change. Therefore, we have compared our results in each group with rats on stock diet at the same weight.

⁸ Addis, T., *Proc. 6th Pacific Science Congress*, 1939, 6, 677.

Summary. 1. Drastic changes in protein, fluid, and salt consumption do not produce striking changes in blood and plasma volumes of the rat, over the periods studied. 2. Increased fluid consumption produced an increase of about 10% in total blood volume and plasma volume at the end of 17 hours, only when salt consumption was simultaneously increased. 3. A high protein diet increased blood and plasma volumes slightly at the end of 7 days, but did not appreciably alter the serum protein concentration. 4. All protein-deficient diets reduced the total circulating protein by 15-20% at the end of 7 days.

16251

Effect of Lipotropic Agents in a Diet Containing Pure Amino Acids in Place of Protein.

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In an experiment where an amino acid mixture replaced casein in a diet deficient in lipotropic factors, Rose, Machella, and György¹ found no appreciable effect of methionine fed to rats at a level of 50 mg per day or equivalent to about 0.8% of the diet. It was not immediately possible to extend this work but the need to determine whether more methionine would be effective or whether some other factor was involved was recognized. In the present study the effect of methionine in the original and in larger dosage was compared with that of choline.

Experimental. The experimental diet and the procedure followed were as previously described. Male albino rats, weighing 150 to 165 g were divided into 4 groups of which one received basal diet alone, the others receiving in addition respectively, 50 mg of methionine, 100 mg of methionine and 25 mg of choline chloride per day in aqueous solution. For about three-fourths of the animals we followed our usual procedure of giving the

supplement in a Caster Cup. Since some of the rats disliked the solution of methionine a stomach tube was used for supplementing those animals which started later in the experiment (equal numbers of all groups). The animals were sacrificed at the end of 3 weeks and the liver fat determined.

Results and Discussion. The experimental data are summarized in Table I. As previously observed¹ the basal diet was quite inadequate in maintaining the weight of the animals and addition of the sulfur containing amino acid neither improved the nutritive value of the ration in this regard to any great extent, nor encouraged appetite. Choline was equally without effect on these factors. Food intake was remarkably constant in all of the groups but much lower than in the previous experiment. The average consumption was 4.1 g per day as compared with 5.9 g in the earlier study.

In the control animals the low food intake and weight loss did not prevent the development of fatty livers, an average value of 20.4% being found. A greater effect of methionine was observed in this experiment than in that previously reported. There was

* The authors are grateful to Miss Caroline Perna for technical assistance.

¹ Rose, C. S., Machella, T. E., and György, P., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 352.

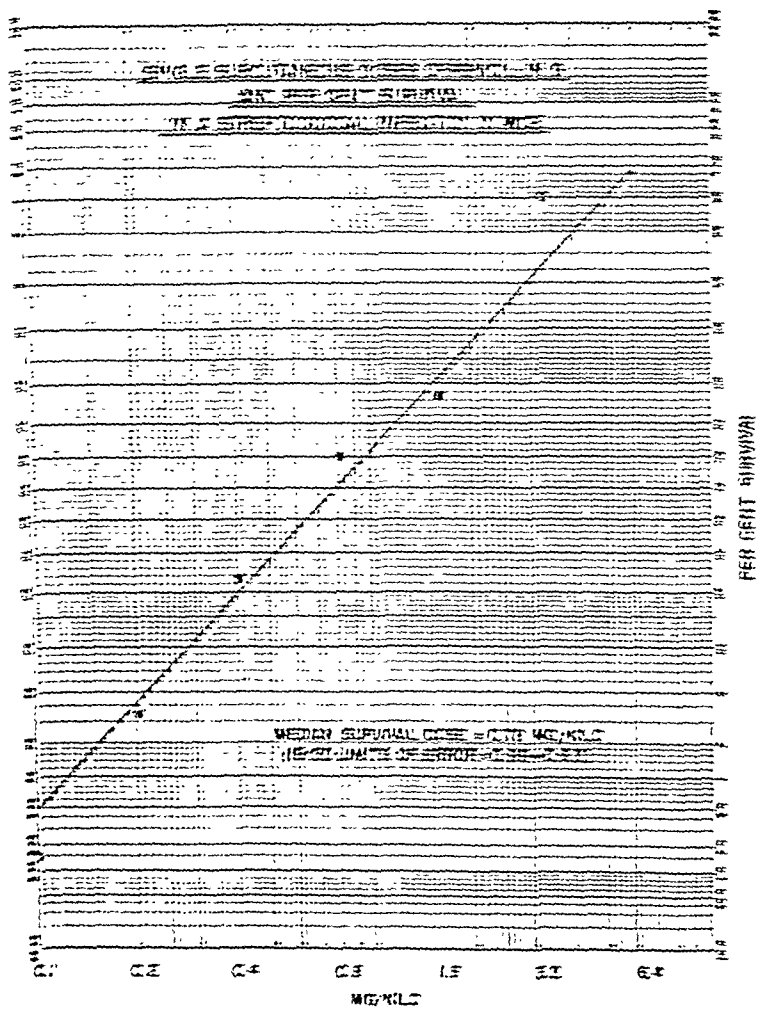


FIG. 1

Single subcutaneous doses of penicillin G plotted against % survival on logarithmic probability paper.

TABLE II

Single Oral and Subcutaneous Doses of Penicillin X

Treatment: Cephaline penicillin X administered as single dose immediately after infection: as 1.5 cc. of aqueous sol. by stomach tube, or as 1.5 cc. subcut.

Single dose mg/kg	Orally Alive Time*	Subcutaneous- Alive Time*
1.5	11.0	11.0
3.0	11.0	11.0
4.5	11.0	11.0
6.0	11.0	11.0
7.5	11.0	11.0
9.0	11.0	11.0

* Survival in 24 hours after infection, expressed as number of mice alive out of total number tested.

(2) 75% of total dose at 0 hour, the other 25% 12 hours later; (3) 75% of total dose at 0 hour and 25% each at 6, 12, and 18 hours later; (4) 75% of total dose at 0 hour and 25% each at 3, 6, 9, 12, 15, 18, and 21 hours later. These 4 schedules were used for each of 5 different total amounts of penicillin X in one experiment, with 20 mice on each schedule for each amount, making a total of 20 dosage groups, each of 20 mice. Two separate experiments of this kind confirmed each other and the pooled results are given in Table III.

It is evident that no advantage was gained by splitting any amount of penicillin X into

TABLE I.

Single Oral and Subcutaneous Doses of Penicillin G.

Treatment: Penicillin G sample containing 650 units per mg; administered as a single dose immediately after infection; in 0.5 cc vol. of aqueous sol. by stomach tube, or in 0.2 cc vol. subcutaneously.

Single dose* mg/kg	Oral Alive/Total†	Subcutaneous Alive/Total‡
25.6	30/30	
12.8	28/30	
6.4	24/30	
3.2	23/30	30/30
1.6	5/30	23/30
0.8	0/30	18/30
0.4		7/30
0.2		1/30
0.1		0/30

	Oral mg/kg	Subcutaneous mg/kg	Oral:Subcutaneous dosage ratio
Median survival dose (19/20 limits of error)‡	2.9 (3.8-2.2)	0.78 (0.99-0.62)	3.7 (5.4-2.5)

* Dose expressed as pure penicillin G containing 1667 units per mg.

† Survival on 21st day after infection, expressed as number of mice alive out of total number tested.

‡ Interpreted as meaning that the chances are 19 to 1 that the true values fall within the indicated limits.

injections at 2- or 3-hour intervals.²

Methods. Our experimental infection was produced by intraperitoneal inoculation of 0.5 cc of a 10^{-5} broth dilution of a 6-hour blood broth culture of *Hemolytic streptococcus*, Group A, strain C 203, in Vanderwerken white mice of 18-22 g weight. The number of organisms in the infecting dose averaged 4000 ± 1000 ; virulence titrations indicated that a single organism (or chain), as determined by colony count, constituted a lethal dose. In the present studies, 80 of 80 untreated control animals died within 48 hours after infection. Treated animals alive on the 21st day after infection are considered to be cured.³

Tests with penicillin G were carried out with the 650 unit per mg sample previously used;¹ crystalline penicillin X was kindly supplied by E. F. Williams of the Physics Division of these Laboratories. In each case, the penicillin was dissolved in sterile distilled

water and the solutions were used without filtration.

Results. The results of single dose treatment with penicillin G are given in Table I. By use of a simplified method for the quantitative evaluation of dosage-response data, devised by Wilcoxon and Litchfield⁴ and illustrated in Fig. 1, the Median Survival Dose (SD_{50}) for subcutaneous administration is estimated to be 0.78 mg per kg, with 19/20 limits of error of 0.99-0.62. The corresponding values for oral administration are 2.9 and 3.8-2.2 mg per kg. The ratio of oral to subcutaneous dosage for penicillin G is thus 3.7, with 19/20 limits of error of 5.4-2.5.

In Table II, results with single doses of penicillin X are given. In this case, it is evident that subcutaneous is about 8 times as effective, therapeutically, as oral administration.

The question of single, as against divided, dosage was investigated by comparing the therapeutic effectiveness of an amount of penicillin given according to each of 4 dosage schedules, as follows: (1) total amount as single dose immediately after infection (0 hr);

² Dowling, H. F., Rotman-Kavka, G., Hussey, H., and Hirsh, H. L., *Am. J. Med. Sci.*, 1947, **213**, 413.

³ Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1939, **67**, 437.

⁴ Wilcoxon, F., and Litchfield, J. T., Jr., to be published.

ment with the early work of MacLeod and Stone⁷ who showed that mice infected with strains of pneumococcal types I, II, or III could be effectively treated with penicillin when the total daily dose (for each of 4 days) was restricted to only 3 injections within an 8-hour period. It is of interest to note, also, that Tillett, McCormack, and Cambier⁸ have concluded that in the treatment of pneumococcal pneumonia with penicillin, repetition of injections continuously throughout each 24-hour period was in many cases not necessary or advantageous. Further experimental evidence indicating that there is no advantage in splitting daily penicillin dosage into a series of small frequently repeated doses has recently been reported by Zubrod⁹ who investigated penicillins G and K in mice under conditions similar to those of the present report.

In the present investigation, the comparison of single and divided dosage schedules has been confined to a maximum treatment period

of only 24 hours. That different results may be obtained under other experimental conditions, is indicated by the data of MacLeod and Stone.⁷ For example, these workers found that treatment with penicillin for a 4-day period could be much more effective than giving an equivalent amount of drug for a single day. In mice infected with a type I pneumococcal strain, recovery of only 74% could be obtained with a total of 600 Oxford units when treatment was restricted to a single day, whereas, recovery of 100% resulted from treatment with a total of only 60 units divided into 4 daily amounts of 15 units each. With type II pneumococcus, about the same degree of recovery (60 to 70%) was produced by a total of 300 units, regardless of whether it was administered in a single day or divided into 4 daily amounts of 75 units each.

Conclusion. From 4 to 8 times as much penicillin was needed for oral, as for subcutaneous administration, to obtain the same degree of therapeutic effect in a streptococcal infection in mice.

In this infection, the administration of daily penicillin dosage in a series of small equally spaced doses was not more effective than giving an equivalent amount of drug in 1 or 2 doses per day.

⁷ MacLeod, C. M., and Stone, E. R., *Bull. N. Y. Acad. Med.*, 1945, **21**, 375.

⁸ Tillett, W. S., McCormack, J. E., and Cambier, M. J., *J. Clin. Invest.*, 1945, **24**, 589.

⁹ Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

16253

Effect of Passive Sensitization and Anaphylactic Shock on Rabbit Bone Marrow.*

ROBERT A. GOOD. (Introduced by Berry Campbell.)

From the Department of Anatomy, University of Minnesota Medical School.

Concepts relating the plasma cell to hyperglobulinemia, globulin formation, immune mechanisms, hypersensitivity, and anaphylactic shock have a firm basis in both clinical and

experimental literature.¹⁻¹⁰ Beginning with

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Maciesca-Jelenska, S., *Munch. Med. Wchnschr.*, 1907, **54**, 1790.

² Downey, H., *Folia haematol.*, 1911, **11**, 275.

³ Huebschmann, P., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, **16**, 110.

⁴ Klein, Z. *f. d. ges. Neurol. u. Psychiat.*, 1914, **21**, 242.

⁵ Oeller, H., *Deutsche. Med. Wchnschr.*, 1923, **49**, 1257.

⁶ Siegmund, H., *Verhandl. d. deutsch. path. Gesellsch.*, 1923, **10**, 114.

⁷ Epstein, E., *Firch. Arch. f. path. Anat.*, 1929, **273**, 89.

⁸ Mas y Magro, F., *Arch. f. Exp. Zellforsch.*, 1929, **8**, 415.

TABLE III.

Single and Divided Doses of Penicillin X.

Treatment: Crystalline penicillin X; 0.2 cc vol. of aqueous sol. administered subcut. on dosage schedules indicated below; each dosage schedule began immediately after infection and was terminated within 24 hours.

Amt of each dose mg/kg	Number of doses			
	One (At time of infection)	Two (At 12-hour intervals)	Four (At 6-hour intervals)	Eight (At 3-hour intervals)
1.0	95*	---	---	---
1/2	98	98	---	---
1/4	58	95	98	---
1/8	48	73	98	100
1/16	25	48	78	100
1/32	---	13	18	35
1/64	---	---	3	8
1/128	---	---	---	8

* Percentage survival on 21st day after infection; 40 mice were used on each of the 20 dosage schedules.

TABLE IV.

Median Survival Doses for Penicillin X Administered by 4 Different Schedules.

Daily dosage schedule (Start: Immediately after infection)		Median survival dosage* mg/kg		
Frequency	No. of doses	Each dose	Total dose	19/20 Limits of error†
Once	1	.13	.13	.43-.04
q. 12 hr	2	.07	.14	.18-.11
q. 6 hr	4	.04	.16	.22-.15
q. 3 hr	8	.03	.24	.32-.21

* These values were obtained from dosage-response lines the slopes of which did not differ from each other significantly.

† The chances are 19 to 1 that the true median survival doses lie within these limits.

several small doses. For example, 8 doses of $\frac{1}{8}$ mg per kg each were no more effective than a single dose of 1 mg per kg; and 8 doses of $\frac{1}{64}$ mg per kg were not as effective as a single dose of $\frac{1}{8}$ mg per kg.

As one would expect, 8 doses of $\frac{1}{8}$ mg per kg each were more effective than 1 dose of $\frac{1}{8}$ mg per kg; and, similarly, 8 doses of $\frac{1}{16}$ each were more effective than a single dose of $\frac{1}{16}$ mg per kg. However, multiple doses of $\frac{1}{2}$ mg per kg could not improve on a single dose of $\frac{1}{2}$ mg per kg since the maximum effect was obtained with the latter dose.

A quantitative comparison of the different dosage schedules may be based upon the Median Survival Dose for each schedule, as calculated from the dosage-response data in Table III. These values, together with their limits of error, are given in Table IV.

Discussion. The greater efficiency of subcutaneous over oral administration of peni-

cillin, as measured by the relative amount of drug needed to produce the same degree of therapeutic effect (Tables I and II) confirms the well-known fact that about 5 times as much oral as subcutaneous dosage is needed to maintain a given concentration of the drug in the blood.^{5,6} The choice between oral and parenteral dosage for clinical use would thus appear to depend upon such factors as relative cost and convenience to the patient.

The results obtained on single versus divided dosage (Tables III and IV) indicate that the total amount of penicillin X for a single day of treatment could be given in 1 or 2 doses, instead of 4 or 8 doses, without significant loss of protection. This is in agree-

⁵ McDermott, W., Bunn, P. A., Benoit, M., DuBois, R., and Reynolds, M. E., *J. Clin. Invest.*, 1946, **25**, 190.

⁶ Hoffman, W. S., and Volini, I. F., *Am. J. Med. Sci.*, 1947, **213**, 513.

TABLE II.*

No. 459	Cells	Plasma cells	Plasmacellular reticulum cells	Reticulum cells
Effect of Active Sensitization and Shock on Bone Marrow.				
Control	1000	9.50	2	9.75
Pre-shock	1000	15.50	37.4	8.20
24 hrs post shock	1000	34.20	10.5	9.20
4 days post shock	1000	36.20	11.75	10.50
Effect of Passive Sensitization and Shock on Bone Marrow.				
No. 1058				
Control	1000	5.25	1	9.50
Pre-shock	1000	9.0	2.5	10.2
24 hrs post shock	1000	6.7	1.5	7.2
4 days post shock	1000	6.2	2.5	1.0
No. 1055				
Control	1000	7.0	5.5	12.2
Pre-shock	1000	8.5	5.25	13.5
24 hrs post shock	1000	6.5	2.75	14.5
4 days post shock	1000	7.75	4.25	11.50

* Each figure is the mean of four 1000 cell counts.

method of Kolouch.¹² Each rabbit was given an injection of 15-30 cc of rabbit serum obtained from the actively sensitized rabbits described above. Eighteen hours later imprints were made of the bone marrow. Six hours later (24 hours after the injection of serum from the hypersensitive rabbits) each rabbit was injected with .4 cc egg white intravenously. All animals save one showed some degree of anaphylactic shock.

Each animal which showed shock was subjected to rib biopsy 8, 24, and 96 hours after

the shock, and a large number of 1000-cell differential counts was made.

Results. The effect of passive sensitization and shock on bone marrow may be compared to that of active sensitization and shock in Table II.

While in active sensitization numerous plasmacellular reticulum cells flooded the bone marrow and following anaphylactic shock the bone marrow became filled with true Marchalko plasma cells, in passively sensitized and shocked rabbits no comparable changes were to be noted. The photo-micrograph, Fig. 1, illustrates this relationship. It is apparent that the cytomorphic cycle culminating in bone marrow plasmacytosis following active immunization or active sensitization and shock is due to factors other than reticular reaction secondary either to the presence of altered globulin in the form of antibody protein in the blood or tissues or factors related to the local or general effect of anaphylactic shock *per se*.

Summary. Studies of serial bone marrow biopsies of passively sensitized and shocked animals reveal: 1. Passive sensitization to a foreign protein does not activate the reticulum as does active sensitization. 2. Anaphylactic shock in passively sensitized rabbits is not followed by the development of bone marrow plasmacytosis as is the case with anaphylactic shock in actively sensitized animals.

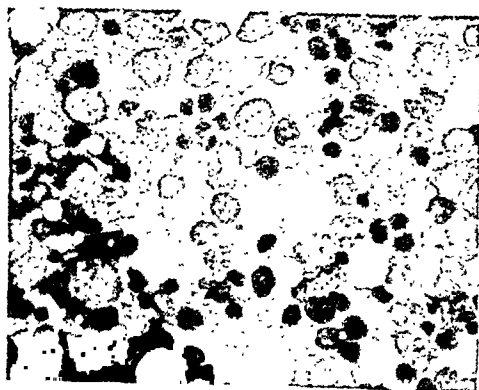


Fig. 1.

Bone marrow imprint from passively sensitized rabbit illustrating the presence of normal bone marrow. Note lack of plasma cells and plasmacellular reticulum cells. Cf. Kolouch, Good, and Campbell,²⁰ Fig. 2.

the interest of Bing and Plum¹¹ in the relationship of bone marrow plasmacytosis to hyperglobulinemia there has accumulated a recent literature which clearly demonstrates that in man and animals reticular organ plasmacytosis occurs concomitant to the development of hyperglobulinemia in a great variety of conditions. Kolouch,^{12,13} studying myelograms of animals sensitized to and shocked with bacterial antigens, discovered a cytomorphic cycle initiated by sensitization and fired by anaphylactic shock that led to bone marrow plasmacytosis. The development of plasma cells, from the plasmacellular reticulum of Rohr,¹⁴ Kolouch found to be associated with rising serum antibody titer. Since this publication, a number of investigators have become interested in the plasma cell of reticular organs and experiments have been performed which indicate that this cell is the source of abnormal globulin and especially antibody globulin.¹⁵⁻¹⁹ In a series of experiments Kolouch, Good, and Campbell²⁰ clarified the morphological mechanisms of reticular organ plasma cell genesis as previously pointed out by Kolouch and showed that the plasma cell content of bone marrow and spleen could be increased following active sensitization and shock with simple protein antigen (egg white) as well as with bacterial antigen. The phenomenon is one, then, of general

hypersensitivity and shock and is not limited to bacterial antigens.

The possible relations of plasmacytosis to passive sensitization and shock have never been investigated. Bjoerneboe and Gormsen¹⁷ have shown that passively induced hyperglobulinemia does not bring about reticular organ plasmacytosis. Bjoerneboe, Gormsen and Lundquist²¹ as well as Fagraeus¹⁸ also conducted experiments which seemed to indicate that increased plasma cell formation does not occur during heightened normal globulin formation. Further experiments carried out in our laboratory²² indicate that local plasma cell formation, under some circumstances at least, is a function of tissue hypersensitivity and antigen-antibody interaction. In these experiments the plasma cells in the acute inflammatory reaction were shown to be a cytological accompaniment of hyperergic inflammation.

In order to test whether or not plasma cell formation is secondary either to passive sensitization and shock the following experiments were performed. Twenty-four young adult albino rabbits were actively sensitized to a simple protein antigen by injecting egg white on alternate days in the following dosages: 1 cc i.v., .5 cc i.v., and 1 cc i.m. A period of 18-30 days was then allowed to elapse before these rabbits were used as blood donors in the following experiment. Blood was obtained by cardiac puncture under aseptic precautions and allowed to clot. The antibody-rich serum was then separated carefully from the clot and that from several donors pooled to provide the serum used for the passive sensitization.

Six young adult albino rabbits weighing 4.0 to 5.3 lb were used for this experiment. Bone marrow biopsies were made after the

TABLE I.

No.	1055	severe	shock
"	1058	moderate	"
"	1057	mild	"
"	1061	moderate	"
"	1062	mild	"
"	1063	no	"

²¹ Bjoerneboe, M., Gormsen, H., and Lundquist, Fr., *J. Immunol.*, 1947, **55**, 121.

²² Good, R. A., unpublished M.S.

⁹ Miller, F. R., *J. Exp. Med.*, 1931, **54**, 333.

¹⁰ Perlzweig, W. A., Delrue, G., and Geschickter, C., *J. A. M. A.*, 1928, **90**, 755.

¹¹ Bing, J., and Plum, P., *Acta Med. Scandinav.*, 1937, **92**, 415.

¹² Kolouch, F., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 147.

¹³ Kolouch, F., Thesis U. of M., 1938.

¹⁴ Rohr, K., *Folia haematol.*, 1936, **55**, 305.

¹⁵ Bjoerneboe, M., and Gormsen, H., *Nordisk Medicin*, 1941, **9**, 891.

¹⁶ Bjoerneboe, M., and Gormsen, H., *Nordisk Medicin*, 1943, **19**, 1155.

¹⁷ Bjoerneboe, M., and Gormsen, H., *Acta Path. et microbiol. Scand.*, 1943, **20**, 649.

¹⁸ Fagraeus, A., *Nordisk Medicin*, 1944, **23**, 1580.

¹⁹ Fagraeus, A., *Nordisk Medicin*, 1946, **30**, 1381.

²⁰ Kolouch, F., Good, R. A., and Campbell, B., *J. Lab. and Clin. Med.*, 1947, **32**, 749.

TABLE I.
Estimated Hepatic Blood Flow (EHBF) in Patients with Hepatic Cirrhosis.

Subject	Age	Sex	Peripheral venous plasma concentration BSP, mg/100 cc	Change in peripheral venous concentration BSP, mg/100 cc/min.	Hepatic extraction* BSP, %	Total BSP removal, mg/min.	EHBF, ml/min.
Laennec's Cirrhosis, History of Chronic Alcoholism.							
I.C.	46	F	3.74	.000	12.0	2.39	850
W.P.	49	M	1.31	+ .005	22.2	1.46	876
R.W.	54	M	2.53	— .005	18.7	2.20	932
E.Me.	67	F	2.15	+ .005	15.8	1.85	983
R.B.	40	M	1.32	+ .006	36.4	2.76	990
J.C.	61	M	1.48	+ .005	21.4	2.72	1320
L.F.	56	M	0.86	.000	24.0	2.16	1690
F.F.	43	M	0.48	— .002	42.5	1.91	1735
D.C.	39	F	1.55	+ .010	10.3	1.85	1906
O.H.	38	M	1.09	.000	22.9	2.54	1995
D.M.†	66	M	1.96	.000	3.4	2.06	4020
Laennec's Cirrhosis, Without History of Alcoholism.							
K.N.†	38	F	1.85	+ .026	11.9	1.35	1026
K.M.	46	F	2.07	+ .003	5.5	1.87	2325
B.M.†	46	M	0.83	+ .002	8.4	2.28	4620
Schistosomiasis—Periportal Fibrosis.							
M.D.†	34	M	0.72	.000	59.4	2.00	842
Biliary Cirrhosis (Hanot's)—Periportal Fibrosis.							
F.G.†	52	F	1.90	+ .010	12.1	1.45	1025
Syphilitic Cirrhosis—Hepar Lobatum.							
J.L.†	49	M	3.00	.000	18.4	1.71	492

* Hepatic extraction of Bromsulfalein was calculated as the difference between peripheral and hepatic venous concentrations divided by the peripheral venous concentration.

† Diagnosis proved by biopsy or necropsy.

TABLE II.
Oxygen Content and Capacity of Peripheral Arterial and Hepatic Venous Blood in Patient with Hepatic Cirrhosis.

Subject	Arterial		Hepatic venous		Oxygen Arterio-venous difference, vol.
	Oxygen content, vol. %	Oxygen saturation, %	Oxygen content, vol. %	Oxygen saturation, %	
Laennec's Cirrhosis with History of Chronic Alcoholism.					
A.A.	14.0	89.6	8.3	52.6	5.7
R.W.	19.4	94.6	13.9	67.5	5.5
F.F.	17.4	95.0	12.0	65.2	5.4
D.C.	14.6	96.7	9.5	62.4	5.1
D.M.	7.3	84.5	6.0	69.0	1.3
Laennec's Cirrhosis Without History of Alcoholism.					
K.N.	15.9	93.4	12.5	73.2	3.4
B.M.	11.8	100.0	6.6	55.2	5.2
Schistosomiasis—Periportal Fibrosis.					
M.D.	14.8	96.0	7.8	50.0	7.0
Biliary Cirrhosis (Hanot's)—Periportal Fibrosis.					
F.G.	15.6	96.2	10.8	66.2	4.8
Syphilitic Cirrhosis—Hepar Lobatum.					
J.L.	14.3	95.9	8.6	57.1	5.7

with alcoholic cirrhosis exhibited a low A-V difference, our results do not support Dock's² suggestion that arterial flow tends to be greater in alcoholic than in non-alcoholic cirrhosis.

Summary. The hepatic blood flow tends to be low or normal in cirrhosis of the liver, regardless of etiology. The hepatic oxygen A-V difference is usually increased.

Estimated Hepatic Blood Flow and Hepatic Venous Oxygen Content in Cirrhosis of the Liver.

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The hepatic blood flow in patients with cirrhosis is generally believed to be reduced because of destruction and distortion of the liver's vascular bed. Experiments designed to test this belief have been carried out by perfusing cirrhotic livers (obtained at necropsy) with saline solution or kerosene, but the results have been inconsistent. Working with large cirrhotic livers, Herrick¹ and Dock² concluded that the capacity for total blood flow in such livers is only moderately decreased and that the arterial inflow may even exceed the normal value. McIndoe,³ who apparently used small, fibrotic livers, believed that hepatic blood flow is greatly reduced by increased resistance to flow in both hepatic arterial and portal venous systems. We have studied the problem in 14 patients with Laennec's cirrhosis, 11 of them with a history of chronic alcoholism, and 3 patients with cirrhosis due respectively to Schistosomiasis, syphilis, and chronic periportal inflammation (biliary cirrhosis).

Methods. Hepatic blood flow (EHBF) was estimated on the basis of the hepatic Bromsulfalein (BSP) clearance.⁴ Hepatic venous blood samples were obtained by means of a venous catheter⁵ placed in an hepatic vein of the right lobe of the liver under fluoroscopic control. Arterial blood was sampled simultaneously through an indwelling arterial needle from a brachial or femoral artery. Oxygen was determined in blood after the method of Van Slyke and Neill⁶ and BSP was measured colorimetrically.⁴ Oxygen

concentrations were corrected on the basis of average arterial oxygen capacities. The subjects, selected from the wards of the Evans Memorial Hospital, Boston, and the Presbyterian Hospital, New York, were studied in the fasting basal state.

Results and Comment. Estimated hepatic blood flow was found definitely below normal (1000 to 2000 ml per minute) in 7 of 17 subjects, regardless of the etiology of the cirrhosis (Table I). In only 3 was EHBF significantly increased, whereas in 7 patients EHBF lay within the normal range.

The high values for EHBF obtained in K.M., B.M., and D.M. cannot be accepted without qualification, because the difference between the concentrations of BSP in hepatic and peripheral blood was so small that even a minor analytical error could have affected the EHBF significantly. Furthermore, in patients with severely damaged livers, the total amount of BSP removed by the liver may be over-estimated. In normal subjects, the removal is so efficient that any extra-hepatic removal is probably negligible, but in cirrhotics impairment of removal by the liver makes possible a proportionally greater removal extra-hepatically. Thus the error introduced by ascribing extra-hepatic removal of BSP to the liver would operate to yield falsely high values for EHBF. Hence, the preponderance of low values among the patients studied becomes more significant.

The data on oxygen content of hepatic venous blood is best discussed in terms of the arterio-venous difference. In all but 2 cases (see Table II) the oxygen A-V difference was greater than the normal difference between arterial and hepatic venous blood (2.5-4.5 ml %). Since only one of 5 subjects

¹ Herrick, F. C., *J. Exp. Med.*, 1907, **9**, 91.

² Dock, W., *Trans. Assn. Am. Phys.*, 1942, **57**, 302.

³ McIndoe, A. H., *Arch. Path.*, 1928, **5**, 23.

⁴ Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., *J. Clin. Invest.*, 1945, **24**, 890.

⁵ Courmand, A., and Ranges, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 462.

⁶ VanSlyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE I.
Penicillin Resistance Induced in Group A Beta Hemolytic Streptococci by Subculture on Penicillin Medium.

Strain	Number of transfers	Initial penicillin Sensitivity,* u/ml	Final penicillin Sensitivity,* u/ml	Fold change
S ₁	60	.01	.04	4
S ₂	60	.02	.04	2
S ₃	60	.01	.17	17
S ₄	60	.01	.01	0
S ₅	60	.01	.045	4.5
S ₆	60	.01	.04	4
U ₅	60	.01	.06	6
U ₆	60	.01	.17	17
A ₁	60	.01	.04	4
A ₂	60	.01	.04	4
R ₂	56	<.01	.015	>1.5
R ₄	56	<.01	.02	>2
R ₅	56	<.01	.03	>3

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

Penicillin sensitivity is defined as the maximum concentration of penicillin on which bacterial growth occurs on the medium in the trough.

To induce resistance, 5 to 7 strains of streptococci were inoculated in single lines on each blood agar plate of a series. Between 3 and 8 concentrations of penicillin were used in a set for each serial transfer. In the R series, subcultures were made alternately on plain blood infusion broth and blood agar medium containing penicillin for a total of 56 serial transfers on each. In the A, S, T, and U series the confluent bacterial growth on the blood agar plate was emulsified in saline and subcultured directly to a new set of plates; a total of 60 such subcultivations was made in each series. Transfers to the next set of plates were made from colonies on medium with the highest concentration of penicillin permitting growth. The penicillin sensitivity for any transfer was considered to be the highest concentration of penicillin that allowed good growth.

Mouse virulence titrations were made as follows: The organisms were incubated at 37°C for 18 hours in tryptose phosphate broth (Difco) containing 5% normal horse serum. The culture was centrifuged at 2000 RPM for 5 minutes, the supernatant fluid discarded, and the sediment resuspended in a sufficient quantity of normal saline to give a final density equal to the McFarland Standard

No. 1. The average plate colony count with this concentration of organisms was approximately 2×10^9 organisms per ml of the saline suspension. Four- to five-week-old white mice (Germantown strain) were inoculated intracerebrally in pairs with 0.03 ml of the 10^0 to 10^4 dilution of the organisms. For this procedure the mice were lightly anesthetized with ether, the skin and hair sterilized with iodine, and the injection made midway between the right ear and eye at a depth of approximately 4 mm. The infected mice were observed for a period of five days. The LD₅₀ was calculated by the Reed and Muench formula.

A. *Resistance Acquired on Media Containing Penicillin.* Thirteen strains of Group A streptococci were subcultivated serially in an attempt to induce resistance to penicillin. A seventeen-fold rise in penicillin resistance was the maximum change obtained. In only two strains did an increase of this magnitude occur. Eleven organisms showed either no change or only a slightly increased resistance after 56 to 60 transfers onto media containing penicillin. Table I summarizes these results.

B. *Resistance Acquired on Control Media.* Eight sensitive parent strains of Group A streptococci were subcultured serially on penicillin-free blood agar medium. After 60 transfers no appreciable increase in penicillin resistance was found when tested by the penicillin whole plate method or by the ditch

Antibiotic Studies on Beta Hemolytic Streptococci. I. Penicillin Resistance Acquired by Group A Organisms.

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There are many reports on the development *in vitro* of resistance to penicillin by staphylococcus, pneumococcus, and meningococcus, but few on beta hemolytic streptococcus. Two groups of investigators studied a single strain of streptococcus (C203, a Group A, type 1 organism) and reported somewhat different rates of development of resistance. Rake and his co-workers¹ found a 30-fold increase in resistance after 58 transfers in penicillin broth, while Todd *et al.*² noted only a 5-fold increase after 35 subcultures and a 4-fold increase after 78 subcultures in penicillin broth. No references to induced resistance for other strains of Group A streptococci and none for Group B or C organisms were found.

The present study was made to look for differences among strains within the A Group. Those reported in the succeeding two communications were undertaken to compare these with the behavior of Group B and C organisms respectively. Variations in penicillin sensitivity have been shown by organisms of these 3 serological groups.³⁻⁸ The

similarities and differences reported by these investigators were observed by us in a screening test of 203 recently isolated strains of Group A, 16 strains of Group B, and 24 strains of Group C streptococci, using the Fleming⁹ ditch plate method.

Materials and Methods. All the strains of beta hemolytic streptococci were isolated from cultures of the nose or throat obtained from patients with acute upper respiratory disease. None had received penicillin therapy. The organisms were grouped by the Lancefield technic. Bacto heart infusion agar (Difco), containing 5% defibrinated sheep's blood, was used as the solid medium. Veal infusion broth, containing 5% defibrinated sheep's blood, served as the liquid medium. In the earlier series, designated R and A, commercial, amorphous sodium penicillin (Lederle) was used, while in the later series, S, T, and U, crystalline sodium penicillin G (Lilly) was employed. Standardization of these penicillins and the final sensitivity tests were performed with standard sodium penicillin G* with a potency of 1667 units per mg.

The ditch plates used to determine the penicillin sensitivity of streptococci were prepared by removing a central trough from a blood agar plate and replacing it with melted agar containing 5% sheep's blood and varying concentrations of standard penicillin G. When this central strip solidified, heavy inocula of organisms were streaked in rows directly across the plate. The character of the growth on both penicillin and penicillin-free media was observed on these plates. The absolute penicillin sensitivity, however, could not be measured by this technic since penicillin diffused into the agar adjacent to the trough.

¹ a. McKee, C. M., and Houck, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 33; b. Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

² Todd, E. W., Turner, G. S., and Drew, L. G. W., *Brit. Med. J.*, 1945, **2**, 603.

³ Watson, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 65.

⁴ Selbie, F. R., Simon, R. D., and McIntosh, J., *J. Path. and Bact.*, 1945, **57**, 47.

⁵ Springarn, C. L., and King, A. N., *U. S. Nav. Med. Bull.*, 1946, **46**, 239.

⁶ Simmons, R. T., and Christie, R., *M. J. Australia*, 1946, **1**, 349.

⁷ Rantz, L. A., Randall, E., Spink, W. W., and Boisvert, P. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 54.

⁸ Rutherford, R. H., Marquardt, G. H., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.*, 1946, 535.

⁹ Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

* Supplied through the courtesy of Dr. Henry Welch, Chief of the Division of Penicillin Control and Immunology, Food and Drug Administration.

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A. *Resistance Acquired on Media Containing Penicillin.* Thirteen strains of Group A streptococci were subcultivated serially in an attempt to induce resistance to penicillin. A seventeen-fold rise in penicillin resistance was the maximum change obtained. In only two strains did an increase of this magnitude occur. Eleven organisms showed either no change or only a slightly increased resistance after 56 to 60 transfers onto media containing penicillin. Table I summarizes these results.

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TABLE II.

Comparison of the Rates of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

Strain	Mean of first 15 transfers of parent* strain on penicillin medium u/ml	Mean of first 15 transfers of control† strain on penicillin medium u/ml
S ₁	.013	.026
S ₂	.015	.012
S ₃	.011	.013
S ₄	.012	.017
S ₅	.015	.009
S ₆	.011	.011
U ₅	.016	.008
U ₆	.015	.019

* Parent strain is a freshly isolated organism.

† Control strain is the parent strain after 60 transfers on control medium.

plate method.

Because preliminary results suggested that adaptation of the streptococci to growth on artificial medium influenced the development of penicillin resistance, the organisms after 60 subcultures on control medium were changed to penicillin medium for 15 transfers. The mean value for the first 15 subcultures of the parent and the control strains on penicillin medium are given in Table II. From these values it is evident that repeated subculturing on plain blood agar does not facilitate the acquisition of resistance to penicillin for most strains of Group A streptococci.

C. *Loss of Acquired Resistance.* Five strains of streptococci after 56 to 60 transfers on penicillin agar, were subcultured serially on penicillin-free medium in an attempt to restore the original sensitivity. The three strains from the R series (R2, 4, and 5) were transferred daily 25 times and the 2 strains from the A series (A1 and 2), 50 times. At the end of 25 subcultures 4 of the 5 strains had returned to their original level of penicillin sensitivity. After 50 transfers the fifth strain had returned to its original level. Todd and his co-workers² observed this same behavior with a resistant culture of the C203 strain after 76 transfers in plain broth.

Four strains were passed serially by intracerebral inoculation in white mice. All of these streptococci had returned to their

original level of penicillin sensitivity by the 12th passage.

D. *Change in Virulence.* Mouse virulence titrations were performed on 10 of the 13 parent strains, on subcultures of the same strains after 60 transfers on control medium, and on all strains after 56 to 60 transfers on penicillin medium. The results are summarized in Table III. All strains showed a marked loss of mouse virulence after serial transfers on penicillin medium. Most of the strains demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on penicillin medium, five strains were passed intracerebrally 12 times through mice. An increase in virulence occurred in 3 of 5 strains.

E. *Antigenic, Hemolytic, and Colonial Changes.* The serological behavior of the streptococci after 56 to 60 serial transfers on penicillin or penicillin-free medium is given in Table IV. In 6 resistant and 2 control strains (*i. e.*, after repeated transfers on control medium) we were unable to demonstrate the presence of the group specific precipitinogen by the Lancefield technic.

A change in the hemolytic behavior of the streptococci when growing on penicillin medium was observed in 10 of the 13 strains studied. Transient conversion from beta to alpha or gamma type of colony appeared when the organisms were growing on maximal concentrations of penicillin. The same strains showed the normal beta hemolysis on lower concentrations of penicillin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies was evident in a given culture on the same concentration of penicillin. When single colonies of any of these types were picked under a dissecting microscope and subcultured on lower concentrations of penicillin or on plain blood agar, they always showed beta hemolysis. The possibility of contamination was eliminated by regrouping isolated alpha and gamma colonies.

The colonial appearance of resistant strains of streptococci did not differ appreciably from the parent or the control strains. How-

TABLE III.
Mouse Virulence of Group A Streptococci with Acquired Penicillin Resistance.

Strain	Initial LD ₅₀ *	LD ₅₀ * after 60 transfers on		LD ₅₀ * of resistant strain after 12 mouse passages
		control medium	penicillin medium	
S ₁	.0000096 ml	.00012 ml	.0030 ml	.000048 ml
S ₂	.00012	.00048	.015	.00096
S ₃	.00096	.000039	>.03	
S ₄	.000030	.00075	>.03	
S ₅	.000096	.00012	.015	
S ₆	.000030	.000096	>.03	
U ₅	.00048	.00019	>.03	
U ₆	.0012	.00096	>.03	
A ₁	.00096	.0096	.0060	
A ₂	.000030	.0096	.0096	
R ₂			.030	.0096
R ₄			.030	>.03
R ₅			.030	>.03

* LD₅₀ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately 2×10^9 organisms per ml.

TABLE IV.
Group Specificity of Streptococci After 56 to 60 Serial Transfers on Penicillin and Penicillin-free Media.

	Strain													
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	U ₅	U ₆	A ₁	A ₂	R ₂	R ₄	R ₅	
Parent culture	A	A	A	A	A	A	A	A	A	A	A	A	A	
Final control culture	A	A	A	A	A	A	N	A	N	A	—	—	—	
Final penicillin-resistant culture	A	A	A	A	A	A	N	N	A	N	N	N	N	

A = group A.

N = not groups A, B, or C.

— = grouping not performed.

ever, transient changes were noted during the process of serial transfers when organisms were growing on maximal concentrations of penicillin. On these plates the colonies frequently were tiny with narrow zones of alpha or beta hemolysis or with gamma hemolysis. No permanent dissociation from mucoid to matt or matt to glossy types was observed. When heavy inocula were streaked on plates with a concentration of penicillin too high to permit visible colonies, hemolysis in the medium and a disappearance of the surface luster were commonly found. Subcultures from these plates failed to grow.

Discussion and Conclusions. Penicillin resistance in strains of Group A streptococci would be of considerable clinical importance, but there is little evidence that such strains actually produce human infections. No proven instance has been found in a review of the literature. Experimentally, resistance to penicillin can be induced in most strains

by *in vitro* technics. However, the increase in resistance, as compared with that of strains of staphylococci, is relatively slight with a 17-fold rise as the maximum change obtained. Using *in vivo* methods in embryonated eggs and in mice,¹⁰ no alteration in penicillin sensitivity was observed. Furthermore, when resistance is induced, animal virulence falls. A similar loss of animal virulence has been described by Miller and Bohnhoff¹¹ for meningococci and McKee and Houck^{12,13} for the C203 strain of Group A streptococcus, type 3 pneumococcus and staphylococcus.

The change in hemolysis seen in streptococci that are growing on medium containing penicillin may be the result of an alteration by penicillin in the metabolism of the organism. A change in streptolysin O or S must

¹⁰ Unpublished data.

¹¹ Miller, C. P., and Bohnhoff, M., *J. Inf. Dis.*, 1947, **81**, 147.

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S ₅	.015	.009
S ₆	.011	.011
U ₅	.016	.008
U ₆	.015	.019

* Parent strain is a freshly isolated organism.

† Control strain is the parent strain after 60 transfers on control medium.

plate method.

Because preliminary results suggested that adaptation of the streptococci to growth on artificial medium influenced the development of penicillin resistance, the organisms after 60 subcultures on control medium were changed to penicillin medium for 15 transfers. The mean value for the first 15 subcultures of the parent and the control strains on penicillin medium are given in Table II. From these values it is evident that repeated subculturing on plain blood agar does not facilitate the acquisition of resistance to penicillin for most strains of Group A streptococci.

C. *Loss of Acquired Resistance.* Five strains of streptococci after 56 to 60 transfers on penicillin agar, were subcultured serially on penicillin-free medium in an attempt to restore the original sensitivity. The three strains from the R series (R2, 4, and 5) were transferred daily 25 times and the 2 strains from the A series (A1 and 2), 50 times. At the end of 25 subcultures 4 of the 5 strains had returned to their original level of penicillin sensitivity. After 50 transfers the fifth strain had returned to its original level. Todd and his co-workers² observed this same behavior with a resistant culture of the C203 strain after 76 transfers in plain broth.

Four strains were passed serially by intracerebral inoculation in white mice. All of these streptococci had returned to their

original level of penicillin sensitivity by the 12th passage.

D. *Change in Virulence.* Mouse virulence titrations were performed on 10 of the 13 parent strains, on subcultures of the same strains after 60 transfers on control medium, and on all strains after 56 to 60 transfers on penicillin medium. The results are summarized in Table III. All strains showed a marked loss of mouse virulence after serial transfers on penicillin medium. Most of the strains demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on penicillin medium, five strains were passed intracerebrally 12 times through mice. An increase in virulence occurred in 3 of 5 strains.

E. *Antigenic, Hemolytic, and Colonial Changes.* The serological behavior of the streptococci after 56 to 60 serial transfers on penicillin or penicillin-free medium is given in Table IV. In 6 resistant and 2 control strains (*i. e.*, after repeated transfers on control medium) we were unable to demonstrate the presence of the group specific precipitinogen by the Lancefield technic.

A change in the hemolytic behavior of the streptococci when growing on penicillin medium was observed in 10 of the 13 strains studied. Transient conversion from beta to alpha or gamma type of colony appeared when the organisms were growing on maximal concentrations of penicillin. The same strains showed the normal beta hemolysis on lower concentrations of penicillin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies was evident in a given culture on the same concentration of penicillin. When single colonies of any of these types were picked under a dissecting microscope and subcultured on lower concentrations of penicillin or on plain blood agar, they always showed beta hemolysis. The possibility of contamination was eliminated by regrouping isolated alpha and gamma colonies.

The colonial appearance of resistant strains of streptococci did not differ appreciably from the parent or the control strains. How-

TABLE I.
Induced Penicillin Resistance in Group B Beta Hemolytic Streptococci.

Strain	Number of passages	Initial penicillin sensitivity,* u/ml	Final penicillin sensitivity,* u/ml	Fold change
T ₁	60	.03	.34	11
T ₂	60	.03	.65	22
T ₃	60	.03	.65	22
T ₄	60	.03	.55	18
T ₅	60	.03	1.35	45
T ₆	60	.03	.65	22
T ₇	60	.03	.65	22
A ₅	60	.015	.90	60
A ₆	60	.015	.80	53
R ₁	56	.01	1.90	190

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

strain which developed a 190-fold increase in resistance in 56 transfers. However, between each transfer of this organism on penicillin medium it was subcultured in penicillin-free blood broth. With the other nine bacteria, the increase in penicillin resistance by the plate to plate technic varied from 11 to 60-fold in 60 serial transfers.

B. *Acquired Resistance on Control Media.* Seven strains subcultured from the original parent stock were transferred serially on penicillin-free blood agar plates. None of the group B organisms studied showed a change in penicillin sensitivity after 60 transfers, nor did they acquire resistance more rapidly than the parent strain when cultured subsequently on penicillin medium for the 61st to 75th passages. The results are given in Table II. This same behavior was described for Group A streptococci in the preceding communication.⁵

C. *Loss of Acquired Resistance.* Three strains of streptococci, after acquiring penicillin resistance, were subcultured on plain blood agar. The R₁ strain was transferred 25 times, and the A₅ and A₆ strains 50 times. At the end of these serial subcultures there was no loss of the acquired resistance in any of the 3 organisms studied.

Three resistant organisms were passed serially by intracerebral inoculation in white mice for 12 passages. Again there was no loss of acquired resistance to penicillin.

Change in Virulence. Using the method described in the preceding communication,⁵ the mouse virulence studies were performed

TABLE II.
Comparison of the Rates of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

Strain	Mean of first 15 transfers of parent* strain on penicillin medium u/ml	Mean of first 15 transfers of control† strain on penicillin medium u/ml
T ₁	.088	.072
T ₂	.083	.050
T ₃	.120	.051
T ₄	.099	.070
T ₅	.105	.076
T ₆	.094	.059
T ₇	.100	.070

* Parent strain is a freshly isolated organism.

† Control strain is the parent strain after 60 transfers on control medium.

on all 10 strains. The results are summarized in Table III. As with the group A organisms, all strains lost virulence after serial transfers on penicillin medium. The majority showed relatively little change after serial transfers on control medium.

Three resistant strains were passed 12 times through mice to restore the lost pathogenicity. The virulence reappeared in one strain. In 2 strains no significant change occurred.

Antigenic, Hemolytic, and Pigment Changes. The results of the serological grouping of the streptococci before and after serial transfers on penicillin and control media are summarized in Table IV. In 2 resistant and one control strain, it was not possible to demonstrate the presence of the group specific precipitinogen. A majority of the organisms in each classification remained groupable.

also be considered. Further study is necessary to elucidate the nature of this interesting phenomenon.

Summary. 1. Thirteen strains of Group A streptococci were transferred serially on medium containing penicillin in an effort to induce resistance. Two strains developed a 17-fold increase in resistance. Eleven strains either remained unchanged or showed an increase up to only 6-fold at the end of 60 subcultures.

2. No increased resistance was induced in the same organism by serial transfers on control medium.

3. Strains which had acquired resistance were restored to their original level of penicillin sensitivity by serial subculture on

penicillin-free medium or by serial intracerebral passages in mice.

4. A marked loss of virulence for mice was shown by all resistant strains of streptococci. The virulence was restored in 2 strains by passage in normal mice.

5. In about one-half of the resistant strains the group specific antigen could not be demonstrated.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were shown by most strains when growing on maximal concentrations of penicillin.

I wish to acknowledge the valuable technical assistance given in these studies by Ernst Jaffe, Sylvia Simons, Deborah Patinkin, and Robert Goff.

16256

Antibiotic Studies on Beta Hemolytic Streptococci: II. Penicillin Resistance Acquired by Group B Organisms.

HORACE M. GEZON. (Introduced by C. Phillip Miller.)

From the Department of Pediatrics, University of Chicago.

Most strains of group B beta hemolytic streptococci possess a higher degree of initial penicillin resistance than group A organisms. The range reported by 3 groups of investigators^{1,2,3} was 0.0625 to 0.25 units of penicillin per ml of medium. We have found the upper and lower limits of sensitivity to extend from 0.01 to 0.10 unit of penicillin per ml of medium by the Fleming ditch-plate method⁴ for 16 freshly isolated strains of group B streptococci.

The present study was carried out in an effort to determine the rate of development of penicillin resistance *in vitro*. In addition we

were interested in learning whether the antigenic, hemolytic, colonial, and mouse virulence changes found in resistant group A organisms⁵ occurred in group B streptococci.

Results. A. Acquired Resistance on Medium Containing Penicillin. Ten strains of group B streptococci were transferred on increasing concentrations of penicillin. The results are given in Table I. A straight line curve results when the mean of fifteen transfers is plotted on semi-logarithmic paper. The graph of the daily values shows the same general trend but with many irregularities in the curve. These variations can be explained by slight differences in the amount of medium per plate, daily loss of potency of penicillin in stock solution, and errors in judgment in estimating the proper range of penicillin concentrations needed for each transfer.

The maximum change occurred in the R₁

¹ Watson, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 65.

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TABLE I.
Induced Penicillin Resistance in Group B Beta Hemolytic Streptococci.

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T ₂	60	.03	.65	22
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T ₅	60	.03	1.35	45
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strain which developed a 190-fold increase in resistance in 56 transfers. However, between each transfer of this organism on penicillin medium it was subcultured in penicillin-free blood broth. With the other nine bacteria, the increase in penicillin resistance by the plate to plate technic varied from 11 to 60-fold in 60 serial transfers.

B. Acquired Resistance on Control Media. Seven strains subcultured from the original parent stock were transferred serially on penicillin-free blood agar plates. None of the group B organisms studied showed a change in penicillin sensitivity after 60 transfers, nor did they acquire resistance more rapidly than the parent strain when cultured subsequently on penicillin medium for the 61st to 75th passages. The results are given in Table II. This same behavior was described for Group A streptococci in the preceding communication.⁵

C. Loss of Acquired Resistance. Three strains of streptococci, after acquiring penicillin resistance, were subcultured on plain blood agar. The R₁ strain was transferred 25 times, and the A₅ and A₆ strains 50 times. At the end of these serial subcultures there was no loss of the acquired resistance in any of the 3 organisms studied.

Three resistant organisms were passed serially by intracerebral inoculation in white mice for 12 passages. Again there was no loss of acquired resistance to penicillin.

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Three resistant strains were passed 12 times through mice to restore the lost pathogenicity. The virulence reappeared in one strain. In 2 strains no significant change occurred.

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Summary. 1. Thirteen strains of Group A streptococci were transferred serially on medium containing penicillin in an effort to induce resistance. Two strains developed a 17-fold increase in resistance. Eleven strains either remained unchanged or showed an increase up to only 6-fold at the end of 60 subcultures.

2. No increased resistance was induced in the same organism by serial transfers on control medium.

3. Strains which had acquired resistance were restored to their original level of penicillin sensitivity by serial subculture on

penicillin-free medium or by serial intracerebral passages in mice.

4. A marked loss of virulence for mice was shown by all resistant strains of streptococci. The virulence was restored in 2 strains by passage in normal mice.

5. In about one-half of the resistant strains the group specific antigen could not be demonstrated.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were shown by most strains when growing on maximal concentrations of penicillin.

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16256

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The present study was carried out in an effort to determine the rate of development of penicillin resistance *in vitro*. In addition we

were interested in learning whether the antigenic, hemolytic, colonial, and mouse virulence changes found in resistant group A organisms⁵ occurred in group B streptococci.

Results. A. *Acquired Resistance on Medium Containing Penicillin.* Ten strains of group B streptococci were transferred on increasing concentrations of penicillin. The results are given in Table I. A straight line curve results when the mean of fifteen transfers is plotted on semi-logarithmic paper. The graph of the daily values shows the same general trend but with many irregularities in the curve. These variations can be explained by slight differences in the amount of medium per plate, daily loss of potency of penicillin in stock solution, and errors in judgment in estimating the proper range of penicillin concentrations needed for each transfer.

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group specificity in some strains after serial transfers on penicillin medium remain unexplained.

Summary. 1. Ten strains of Group B streptococci were transferred serially on media containing increasing quantities of penicillin in an effort to produce resistance. All 10 organisms showed a significant increase in resistance varying from 11 to 190-fold over the parent strains.

2. No increased resistance was induced by serial transfers on control medium.

3. In contrast to Group A organisms, Group B streptococci did not lose resistance on subculture on blood agar or on serial

passage through mice.

4. Virulence decreased in all resistant strains of streptococci. The pathogenicity was partially restored in one organism but remained unchanged in two after passage in normal mice.

5. In only 2 resistant strains was the group specificity lost.

6. Transient alterations in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

16257

Antibiotic Studies on Beta Hemolytic Streptococci: III. Penicillin Resistance Acquired by Group C Organisms.

HORACE M. GEZON. (Introduced by C. Phillip Miller.)

From the Department of Pediatrics, University of Chicago.

Streptococci of Lancefield Group A or B present group differences as well as strain variations within a group, when transferred serially on media containing penicillin. These characteristics are described in the preceding reports.^{1,2} In general, Group A differ from Group B streptococci in having a lower initial penicillin sensitivity. They show less tendency to acquire penicillin resistance *in vitro*, lose acquired resistance more rapidly on passage in mice or plain medium, and develop colonial and hemolytic changes less quickly when growing on penicillin medium.

The initial penicillin sensitivity of Group C organisms is reported³⁻⁵ to vary from 0.0078 to

0.0313 units of penicillin per ml of medium. We have studied 24 recently isolated strains by the Fleming⁶ ditch-plate method and have found their sensitivity range to be 0.007 to 0.03 units of penicillin per ml of medium.

The present study was undertaken to determine the similarities and differences between streptococci of Groups A, B, and C as regards their ability to acquire penicillin resistance.

Results. Acquired Resistance on Medium Containing Penicillin. Five strains of Group C streptococci underwent serial transfer on blood agar containing penicillin. Three of the 5 organisms after 60 subcultures demonstrated a moderate increase in penicillin resistance varying from 11- to 16-fold. Two forms, U₂ and R₂, after 60 and 56 transfers respectively, developed only a slightly increased resistance. The results are given in Table I.

Acquired Resistance on Control Medium. Four strains of streptococci subcultured from

¹ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 208.

² Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 212.

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PENICILLIN RESISTANCE GROUP B STREPTOCOCCI

TABLE III.
Mouse Virulence of Group B Streptococci with Acquired Penicillin Resistance.

Strain	Initial LD ₅₀ *	LD ₅₀ * after 60 transfers on		LD ₅₀ * of resistant strain after 12 mouse passages
		control medium	penicillin medium	
T ₁	.000048 ml	.0000015 ml	.0096 ml	
T ₂	<.0000030	.000012	>.03	
T ₃	<.0000030	.000060	>.03	
T ₄	.000096	.00030	.015	
T ₅	<.0000030	.0000000015	>.03	>.03 ml
T ₆	<.0000030	.000075	>.03	
T ₇	<.0000030	>.03	.003	.0048
A ₅	.0015	.00096	>.03	
A ₆	.012	.0048	.0096	
R ₁	.00030		>.03	.00048

*LD₅₀ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately 2×10^9 organisms per ml.

TABLE IV.
Group Specificity of Streptococci after 56 to 60 Serial Transfers on Penicillin and Penicillin-Free Media.

	Strain										
	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	A ₅	A ₆	R ₁	
Parent culture	B	B	B	B	B	B	B	B	B	B	
Final control culture	B	B	B	B	B	B	B	N	B	—	
Final penicillin-resistant culture	N	B	B	B	B	B	B	B	B	N	

B = group B.

N = not groups A, B, or C.

— = grouping not performed.

The alteration in the character of the hemolysis described for group A organisms in the preceding report⁵ was even more striking and more permanent for group B streptococci. All 10 strains showed varying degrees of change from beta to alpha or gamma colonies at maximal concentrations of penicillin. Single colonies of either the gamma or alpha types when subcultured onto plain blood agar produced only beta hemolysis. Small colony forms occurred frequently on penicillin medium. These reverted back to the normal forms when transferred to control medium.

Several of the streptococci of Group B showed the presence of the reddish pigment described by Lancefield.⁶ No modification in this pigmentation appeared after the organisms acquired penicillin resistance.

Discussion and Conclusions. All strains of Group B streptococci studied showed a rapid development of penicillin resistance as compared with Group A organisms. The 190-fold

increase demonstrated by one organism after 60 transfers and the 107-fold rise observed in another after 80 subcultures⁷ indicate that great penicillin resistance may be induced in Group B streptococci. We have not yet attained the upper limit of resistance for any of the strains tested.

The greater ease in acquiring penicillin resistance in Group B than in Group A streptococci is probably of clinical importance. An example of this phenomenon was seen recently in a patient who died of subacute bacterial endocarditis at the Albert Merritt Billings Hospital of the University of Chicago. Shortly before death a strain of Group B streptococci was obtained from his pharynx, and at autopsy from his blood. He had received up to 5 million units of penicillin a day for the previous 30 days.

The loss of mouse virulence, changes in hemolytic characteristics, variations in the colonial appearance, and disappearance of

⁶ Lancefield, R. C., *J. Exp. Med.*, 1934, 59, 459.

⁷ Unpublished data.

group specificity in some strains after serial transfers on penicillin medium remain unexplained.

Summary. 1. Ten strains of Group B streptococci were transferred serially on media containing increasing quantities of penicillin in an effort to produce resistance. All 10 organisms showed a significant increase in resistance varying from 11 to 190-fold over the parent strains.

2. No increased resistance was induced by serial transfers on control medium.

3. In contrast to Group A organisms, Group B streptococci did not lose resistance on subculture on blood agar or on serial

passage through mice.

4. Virulence decreased in all resistant strains of streptococci. The pathogenicity was partially restored in one organism but remained unchanged in two after passage in normal mice.

5. In only 2 resistant strains was the group specificity lost.

6. Transient alterations in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

16257

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The initial penicillin sensitivity of Group C organisms is reported³⁻⁵ to vary from 0.0078 to

0.0313 units of penicillin per ml of medium. We have studied 24 recently isolated strains by the Fleming⁶ ditch-plate method and have found their sensitivity range to be 0.007 to 0.03 units of penicillin per ml of medium.

The present study was undertaken to determine the similarities and differences between streptococci of Groups A, B, and C as regards their ability to acquire penicillin resistance.

Results. Acquired Resistance on Medium Containing Penicillin. Five strains of Group C streptococci underwent serial transfer on blood agar containing penicillin. Three of the 5 organisms after 60 subcultures demonstrated a moderate increase in penicillin resistance varying from 11- to 16-fold. Two forms, U₃ and R₃, after 60 and 56 transfers respectively, developed only a slightly increased resistance. The results are given in Table I.

Acquired Resistance on Control Medium. Four strains of streptococci subcultured from

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PENICILLIN RESISTANCE GROUP C STREPTOCOCCI

 TABLE I.
 Induced Penicillin Resistance in Group C Beta Hemolytic Streptococci.

Strain	Number of passages	Initial penicillin sensitivity,* u/ml	Final penicillin sensitivity,* u/ml	Fold change
U ₁	60	.01	.16	16
U ₂	60	.01	.14	14
U ₃	60	.03	.14	4
U ₄	60	.02	.33	16
R ₃	56	<.01	.02	> 2

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

TABLE II.

Comparison of the Rate of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

Strain	Mean of first 15 transfers of parent* strain on penicillin medium u/ml	Mean of first 15 transfers of control strain on penicillin medium u/ml
U ₁	.033	.020
U ₂	.030	.020
U ₃	.033	.016
U ₄	.033	.034

* Parent strain is a freshly isolated organism.

† Control strain is the parent form after 60 transfers on control medium.

the original parent organisms were passed on control (*i.e.*, penicillin-free) blood agar. After 60 transfers these bacteria were retested for penicillin sensitivity by the Fleming ditch-plate method. For purposes of comparison alternate lines on plates containing varying concentrations of penicillin were inoculated with the paired resistant and control organisms. No increased penicillin resistance was demonstrated by these control organisms.

In an effort to discover whether adaptation to growth on artificial medium would influence the development of penicillin resistance,

the control organisms were carried on penicillin medium for the 61st to 75th passages. The mean values for the first 15 transfers of the parent and the control strains on penicillin medium are given in Table II. Again as was found with both Group A and B streptococci, the repeated subcultivation on plain blood agar does not facilitate the acquisition of resistance to penicillin.

Loss of Acquired Resistance. Only one strain, R₃, was grown serially on penicillin-free medium and two strains, U₁ and R₃, passed in untreated mice in an effort to restore the penicillin sensitivity. After 25 transfers on plain blood agar and 12 passages intracerebrally in mice no decrease in the acquired resistance of the organism had occurred.

D. Change in Virulence. Using the method described in the first report of this series,¹ mouse virulence studies were performed on all 5 strains. The results appear in Table III. As was observed previously with Group A and B streptococci, all organisms suffered a marked loss of mouse virulence after repeated subcultivation on penicillin-containing medium and relatively little change after a like number of transfers on control medium.

Two resistant strains were passed 12 times

 TABLE III.
 Mouse Virulence of Group C Streptococci with Acquired Penicillin Resistance.

Strain	Initial LD ₅₀ *	LD ₅₀ * after 60 transfers on		LD ₅₀ * of resistant strain after 12 mouse passages
		control medium	penicillin medium	
U ₁	.00015 ml	.000096 ml	>.03 ml	.006 ml
U ₂	.0018	.0015	>.03	
U ₃	.0000048	.000030	>.03	
U ₄	.000060	.00048	>.03	
R ₃	.000096		>.03	.00096

* LD₅₀ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately 2×10^9 organisms per ml.

TABLE IV.
Group Specificity of Streptococci After 56 to 60 Serial Transfers on Penicillin and Penicillin-Free Media.

	Strain				
	U ₁	U ₂	U ₃	U ₄	R ₃
Parent culture	C	C	C	C	C
Final control culture	C	C	C	C	—
Final penicillin resistant culture	C	N	N	N	C

C = group C.

N = not group A, B, or C.

— = grouping not performed.

in mice in trying to restore the virulence. The LD₅₀ values are given in Table III. In both instances only a partial restoration was accomplished.

Antigenic and Hemolytic Changes. The

results of the serological grouping of the streptococci before and after repeated transfers on penicillin and control media are given in Table IV. In the 4 control organisms studied no loss of the group specific antigen

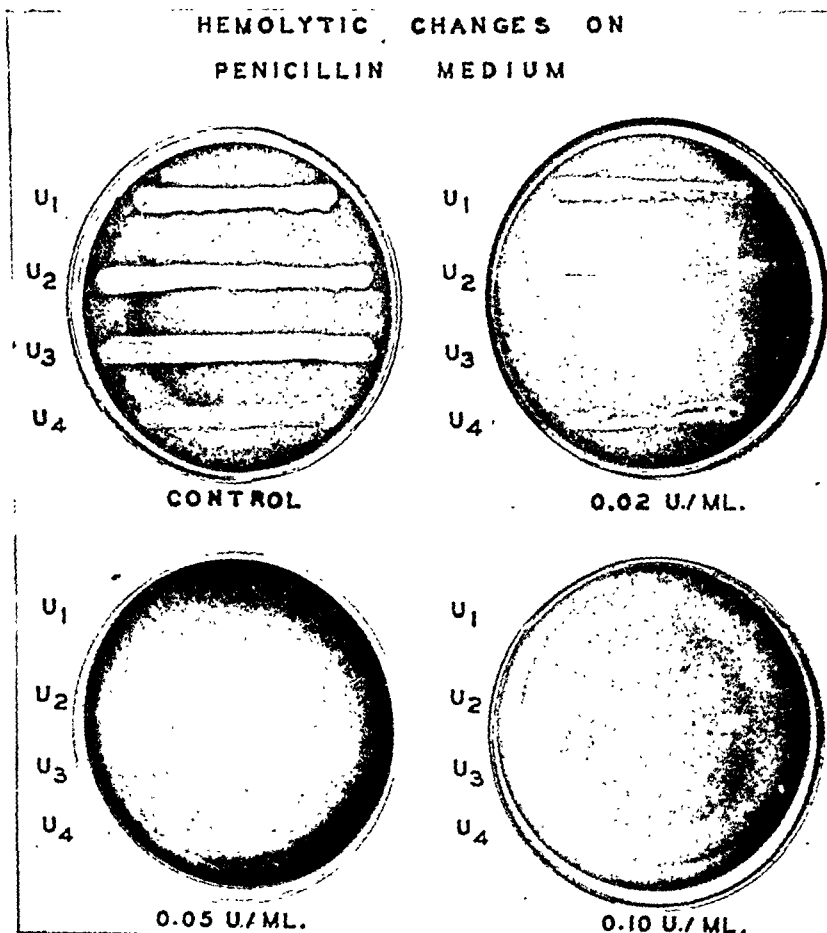


FIG. 1.

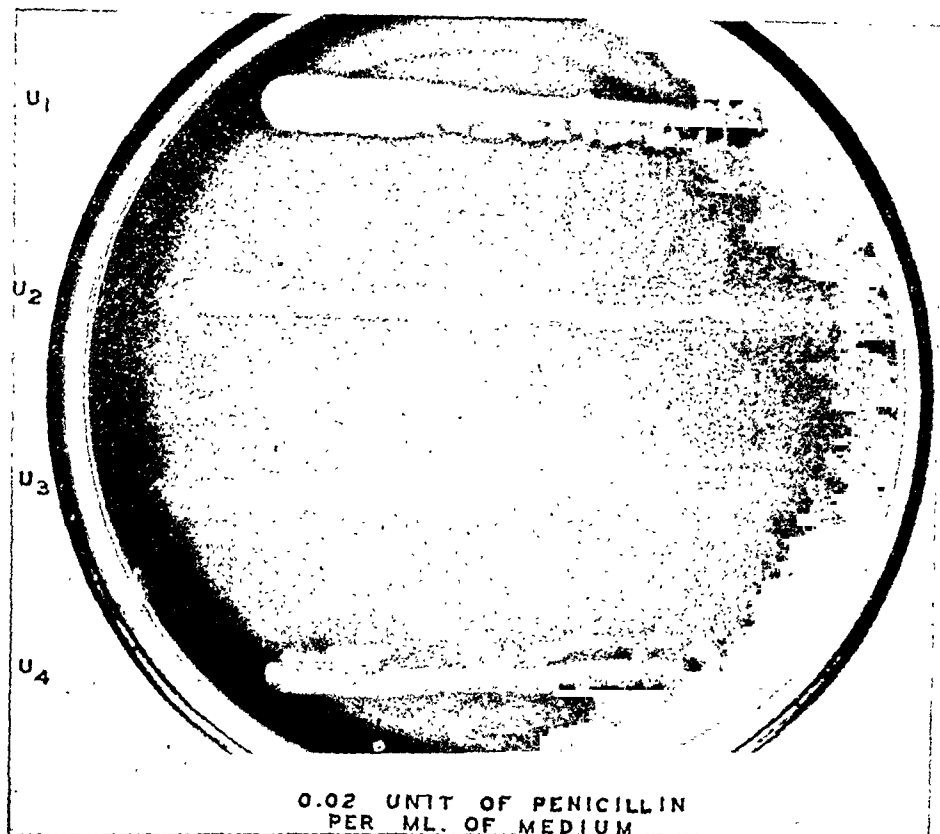


FIG. 2.

occurred. After acquiring penicillin resistance, only 2 of 5 strains were classifiable by the Lancefield technic.

The change in the character of the hemolysis described for Group A and B organisms appeared as well in all 5 strains of Group C streptococci when the organisms were grown on medium containing penicillin. An example of this hemolysis is given in Fig. 1. A close-up view of the 0.02 unit per ml plate of Fig. 1 is shown in Fig. 2. Here the hemolytic changes are best demonstrated by the U_2 strain with both green and clear hemolysis present in the same growth. Frequently the transformation to alpha or gamma types of colonies was present in the maximum concentration of penicillin and also at levels below that which would permit growth. Mixtures of alpha, beta, and gamma hemolytic colonies were seen on plates containing a single concentration of penicillin. Some strains of

Group C streptococci normally show a zone of greenish hemolysis outside the wide clear zone. Forms with this characteristic display an exaggeration of this color when grown on minimal concentrations of penicillin.

Discussion and Conclusions. The behavior of Group C streptococci on penicillin medium resembles in many respects that of Group A and B organisms.^{1,2} The same drop in mouse virulence, transient hemolytic changes, and colonial changes were demonstrated by nearly all strains of the 3 groups. In general, Group B streptococci develop a much higher degree of *in vitro* resistance at a faster rate than either Group A or C organisms. The acquired resistance appears to be a more permanent characteristic of Group B and C organisms than of Group A. Only in the latter did passage in normal mice or on penicillin-free medium restore the sensitivity.

If these *in vitro* studies could lead to the

conclusion that resistance to penicillin in Group A and C streptococci is not likely to develop, it would be of great clinical importance. However, it should be emphasized that these observations on the group and strain behavior of artificially resistant streptococci, while interesting and valid, must not be assumed to apply by analogy to natural resistance.

Summary. 1. Five strains of Group C streptococci were subcultured serially on medium containing penicillin in an effort to induce resistance. Three of the 5 strains developed a 14- to 16-fold increase after 60 transfers. Two strains demonstrated only a 2- to 4-fold increase after similar subcultures.

2. No increased resistance was induced by serial transfers on control medium.

3. Acquired resistant organisms maintained

their resistance on serial subcultures on blood agar medium or on serial passages through mice.

4. Decreased mouse virulence was shown by all resistant strains of streptococci. Relatively little change appeared in the control organisms. Lest virulence was partially restored in 2 strains by passage in normal mice.

5. The group specific precipitinogen was demonstrable in only 2 of the 5 resistant strains, but was present in 4 control organisms.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

16258 P

Propagation of the Mammary Tumor Milk Agent in Tumors from C₅₇ Black Mice.*

JOHN J. BITTNER.

From the Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis, Minn.

If females of the C₅₇ black strain obtained the mammary tumor milk agent by nursing females of cancerous strains, mice of some sublines may, when they are maintained as breeders, show incidences of mammary cancer in excess of 50%¹⁻⁴ although mice of this strain are generally regarded to have a "low susceptibility" for spontaneous mammary

cancer. In 2 studies it was determined that the incidence may be approximately as high,⁴ or even higher,² in the progeny than in the mice of the fostered generation, demonstrating that the females may propagate and transmit the milk agent. Fostered females and their descendants of a line of the C₅₇ black strain which gave a low incidence of spontaneous tumors have also been found to transmit the milk agent.⁵

The milk agent, one of the 3 causative factors for mammary cancer in mice,⁶ may be

* Assisted by grants from the Citizens Aid Society of Minneapolis, the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, the Cancer Research Fund of the University of Minnesota Graduate School, the Minnesota Cancer Society, and the Elsa U. Pardee Foundation.

¹ Bagg, H. J., *International Cancer Research Foundation, Report of Activities During 1933*, p. 14.

² Fekete, E., and Little, C. C., *Cancer Research*, 1942, **2**, 325.

³ Andervont, H. B., *J. Nat. Cancer Inst.*, 1943, **3**, 359.

⁴ Haugensen, C. D., and Randall, H. T., *Cancer Research*, 1945, **5**, 352.

⁵ Bittner, J. J., *J. Nat. Cancer Inst.*, 1949, **1**, 155.

⁶ Bittner, J. J., *Public Health Rep.*, 1939, **54**, 1590.

TABLE I.
Activity of the Milk Agent in Spontaneous Mammary Tumors from C₅₇ Blk. Mice, Line 6.

Tumor No.	Date injected	Gm.Eq. inj.	No.	% Ca	% living
7476 (A ₂)*	6/27/46	1/50	19	79	16
		1/1000	13	46	21
7477 (A ₂)*	"	1/50	29	55	14
		1/1000	25	60	36
7478 (Z ₁)	"	1/100	31	58	23
7788 (Z ₁)	3/26/47	1/50	56	30	67
		1/1000	32	25	69

* Tumors inoculated.

TABLE II.
Comparative Activity of the Milk Agent in Tumors from C₅₇ Blk. Mice.

Tissue extracted	Gm.Eq.inj.	Tumor No. 7476			Tumor No. 7477		
		No.	% Ca	% liv.	No.	% Ca	% liv.
Spontaneous tumors	1/50	19	79	16	29	55	14
	1/1000	13	46	21	25	60	36
Transplants: after 1 passage	1/50	9	67	22	29*	100	—
	1/1000	21	48	29	20	65	25
Transplants: after 10 passages	1/10	38	39	58	38	66	34
	1/1000	44	25	73	44	16	82

* Average cancer age, 371 days.

recovered from extracts of spontaneous mammary cancer⁷⁻⁹ from mice of cancerous strains and has been found to survive for at least 10 passages of these tumors in mice which did not themselves have the milk agent.¹⁰

In this study we have investigated the presence of the milk agent in 4 spontaneous mammary tumors which developed in mice of Line 6 of the C₅₇ black stock. Two tumors, Nos. 7476 and 7477, arose in the progeny of a female which had obtained the milk agent from a foster mother of the cancerous A strain. The mother of the C₅₇ black mice died cancerous. The other tumors, Nos. 7478 and 7788, developed in females which had been nursed by a female of the cancerous C₃H stock. In addition, tumors 7476 and 7477 were transplanted into mice of the C₅₇ black

stock which had not received the milk agent, either from their mother or a foster mother. The transplanted tumors were tested after the first passage and also after the tenth passage for the survival of the milk agent.

The test animals were ZBC mice derived by reciprocal matings between mice of the fostered A and C₃H strains. Controls have been found to have few spontaneous mammary tumors whereas when the ZBC mice obtain the milk agent, high incidences have been observed.¹¹

The tumor extracts were all prepared in the same manner. The tissue was ground in a mortar with sand and suspended in either physiological saline or triple distilled water (1:10 by weight). The suspension was centrifuged for 7 to 10 minutes at approximately 2,500 r.p.m. and 1 cc of the supernatant, at various dilutions, was injected intraperitoneally into the test animals. The injected mice were continued as breeders to insure an adequate hormonal stimulation for the genesis

⁷ Bittner, J. J., *Science*, 1941, **93**, 527.

⁸ Barnum, C. P., Ball, Z. B., Bittner, J. J., and Visscher, M. B., *Science*, 1944, **100**, 575.

⁹ Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **3**, 143.

¹⁰ Bittner, J. J., Evans, C. A., and Green, R. G., *Science*, 1945, **101**, 95.

¹¹ Bittner, J. J., *AAAS, Research Conference on Cancer*, 1945, p. 63.

of mammary cancer.

The data are presented in Tables I and II.

The results demonstrate that the mammary tumor milk agent may be recovered from spontaneous mammary cancers which developed in mice of the C₅₇ black stock and may be propagated in the transplants of these tumors. Two of the tumors arose in mice which had received the agent from their mothers, again demonstrating that females of

the C₅₇ black strain, at least some sublines, may transmit the agent in the milk.

Summary. Mice of some sublines of the C₅₇ black stock may propagate and transmit the mammary tumor agent. The agent may be recovered from spontaneous mammary cancers which developed in mice of the strain and may survive for at least 10 passages of these tumors in mice without the agent.

16259

Effect of Bacterial Endotoxins on Carbohydrate Metabolism of Rabbits.*

ERNEST KUN AND C. PHILLIP MILLER.†

From the Departments of Pharmacology and Medicine, University of Chicago.

The injection into animals of heat-killed cultures of certain bacterial cells has been shown to produce marked hyperglycemia.^{1,2} Diphtheria toxin which has been studied more extensively³ causes a similar rise in blood sugar and depletion of tissue carbohydrate. Some investigators^{4,5} have concluded that diphtheria toxin affects metabolism by damaging the liver, causing increased glycolysis and diminished phosphorolysis. The biochemical mechanism by which the metabolites or cellular constituents of bacteria interfere with the metabolism of the infected animal is not well understood.

The experiments described below concern the effects of meningococcal and *Salmonella* endotoxins on the intermediate carbohydrate

metabolism of rabbits. As in experiments already reported,⁶ the experimental conditions may be defined as acute endotoxin poisoning. The effect of a lethal dose of endotoxin was studied during the short period of intoxication which terminated in the death of the animal in 1.5-3 hours. It is believed that, under such circumstances, the effect of the toxin is not significantly altered by spontaneous changes in metabolism.

Materials and Methods. The endotoxins were sterile, unpurified preparations made from washed bacterial cells by the method already described.⁶ The intravenous dose which was lethal in 2 to 3 hours was 20-50 mg per kg. Rabbits weighing 1.5-3 kg were used. The experimental and control animals were fasted for 24 hours before each experiment. No anesthesia was used. Blood samples were taken by cardiac puncture. Tissue analyses were carried out immediately after the death of the animal. Pairs of rabbits having almost identical weights were kept under the same environmental conditions throughout each experiment. One rabbit in each pair was injected with endotoxin and the other kept as a control and sacrificed when death occurred in the former. In some

* This investigation was undertaken and supported jointly by the U. S. Navy, Office of Naval Research, and the University of Chicago.

† With the technical assistance of Mrs. S. E. Gurnee.

1 Menton, M. L., and Manning, H. M., *J. Med. Res.*, 1924, 44, 675.

2 Zweekwer, L. T., and Goodell, H., *J. Exp. Med.*, 1925a, 42, 43; *J. Exp. Med.*, 1925b, 42, 57.

3 Holmes, E., *Physiol. Rev.*, 1939, 19, 439.

4 Soskin, S., Allweiss, M. D., and Mirsky, J. A., *Arch. Int. Med.*, 1935, 56, 927.

5 Taubenhaus, M., and Soskin, S., *J. Clin. Endocrin.*, 1942, 2, 171.

6 Kun, E., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 197.

instances the animals were sacrificed before death and the same changes were found in the concentration of metabolites and enzyme activities as in the animals which succumbed to endotoxin.

Blood glucose was determined by the method of Nelson,⁷ lactic acid according to Barker and Summerson,⁸ pyruvic acid as described by Elgart and Nelson,⁹ and acid soluble phosphorus by the method of Schneider.¹⁰ Glycogen was determined as glucose.¹¹

Results. The results of the analyses for blood sugar, lactic acid, phosphorus and pyruvic acid are shown in Fig. 1, 2, 3, and 4. The graphs were constructed from data obtained from experiments of identical length.

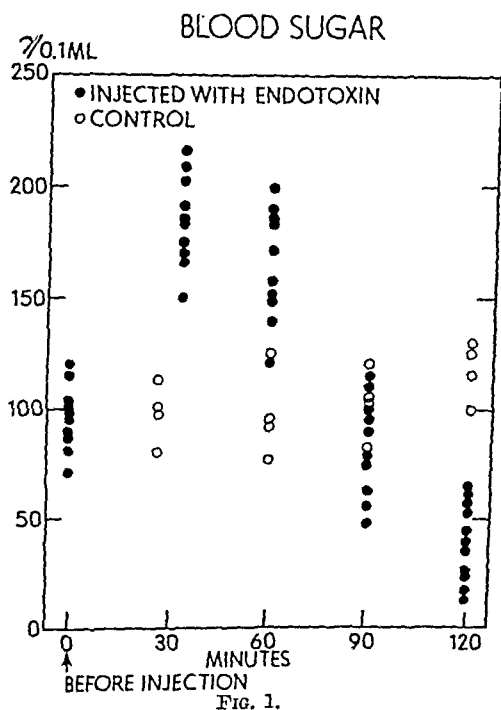


Fig. 1.

⁷ Nelson, N., *J. Biol. Chem.*, 1944, 153, 375.

⁸ Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, 138, 535.

⁹ Elgart, J. S., and Nelson, N., *J. Biol. Chem.*, 1941, 138, 443.

¹⁰ Schneider, W., *J. Biol. Chem.*, 1945, 161, 293.

¹¹ Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, Burgess Publishing Company, 1945.

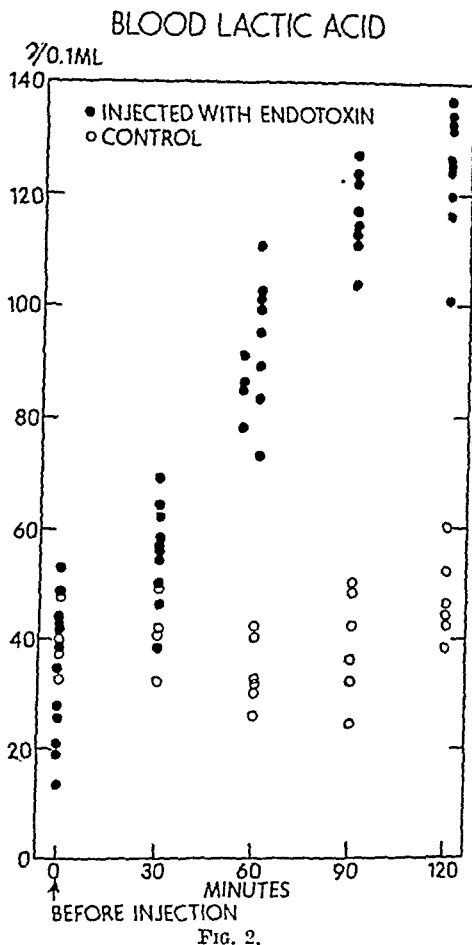


Fig. 2.

Each point represents the blood values of one individual animal.

The results show that the endotoxins caused hyperglycemia which reached its maximum 30 minutes after the injection and was followed by hypoglycemia. Blood lactic acid showed marked increase. Blood phosphorus fell slightly a few minutes after the injection and then rose sharply. The blood pyruvic acid content decreased after the injection of the toxin and remained below the normal level.

The results of the tissue analyses are given in Fig. 5, in which the liver and muscle glycogen, lactic acid and pyruvic acid of normal animals are compared with the results obtained from animals injected with meningococcal endotoxin. (Eight pairs of rabbits were used for glycogen, 5 pairs for tissue

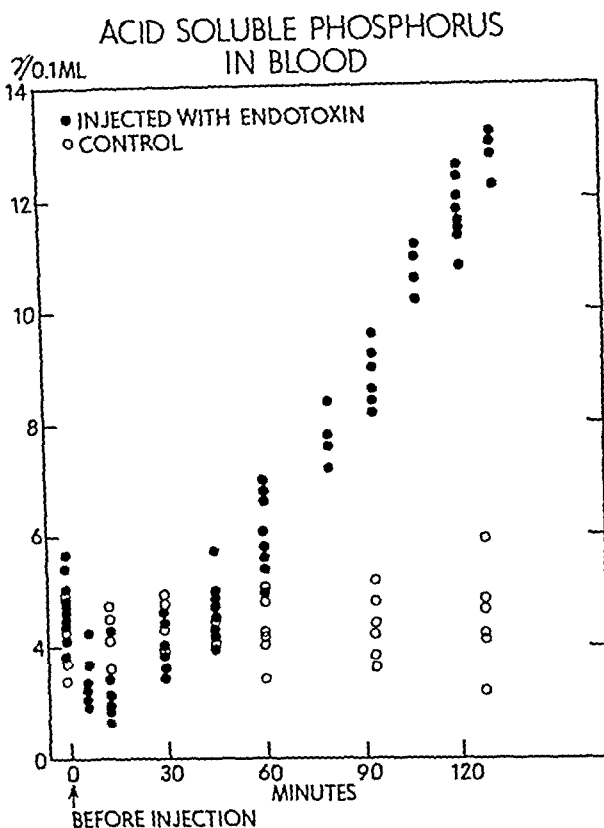


FIG. 8.

lactic acid and 4 pairs for tissue pyruvic acid determinations.)

The greatest difference between the control rabbits and those injected with endotoxin was found in the glycogen content of liver and muscle, particularly in the former, as the livers of the animals poisoned by endotoxin contained only traces of glycogen.

The lactic acid content of the liver and muscle in the endotoxin treated rabbits was markedly increased, whereas the pyruvic acid content was diminished.

It is well established that in mammalian tissue under normal circumstances glycogen is broken down to pyruvate which is further oxidized through various channels. If the aerobic conditions in tissues are altered in some way, accumulation of lactate and inorganic phosphate occurs.¹² Our observations on these constituents in the blood and tissues of our experimental animals suggested, therefore, that the injection of the bacterial endo-

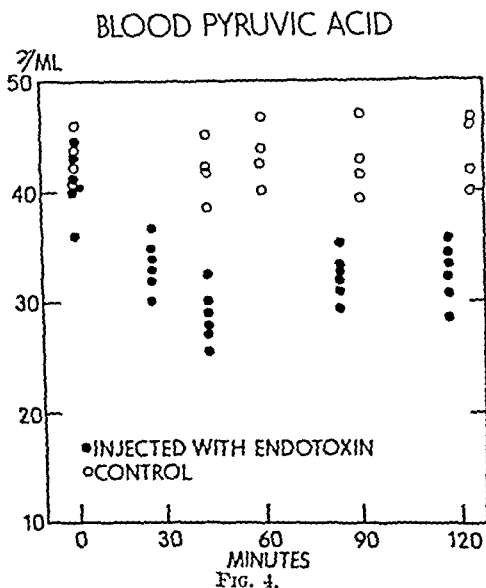


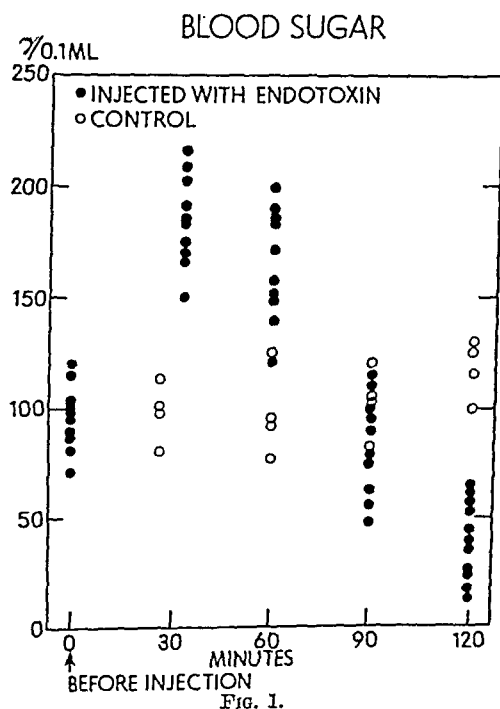
FIG. 9.

¹² Barron, E. S. G., *Advances in Enzymology*, 1943, 3, 149.

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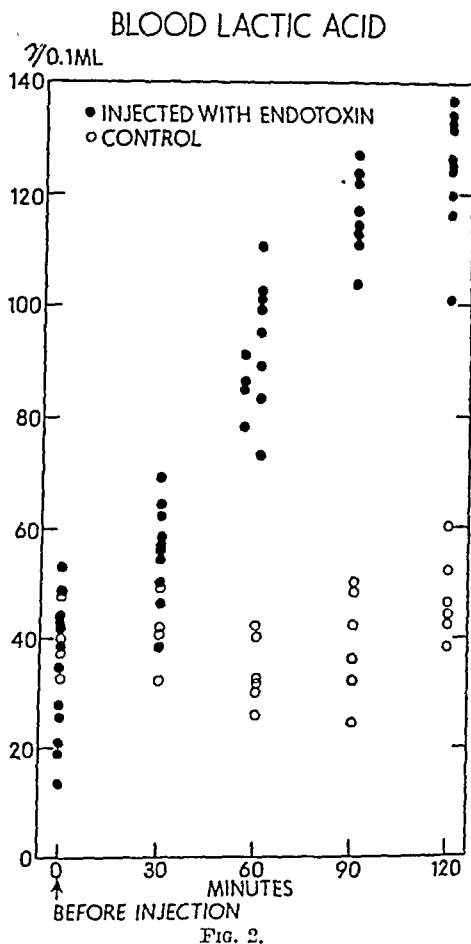
⁷ Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

⁸ Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

⁹ Elgart, J. S., and Nelson, N., *J. Biol. Chem.*, 1941, **138**, 443.

¹⁰ Schneider, W., *J. Biol. Chem.*, 1945, **161**, 293.

¹¹ Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, Burgess Publishing Company, 1945.



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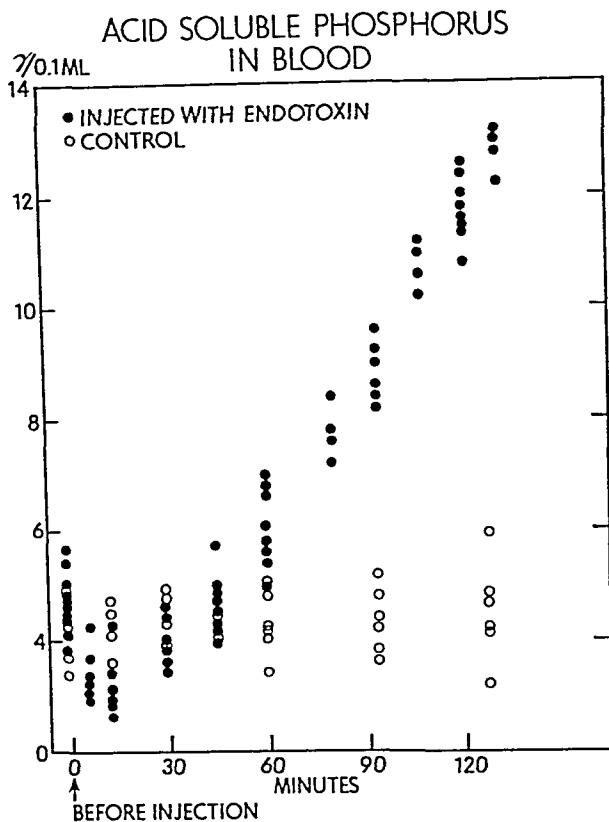


FIG. 3.

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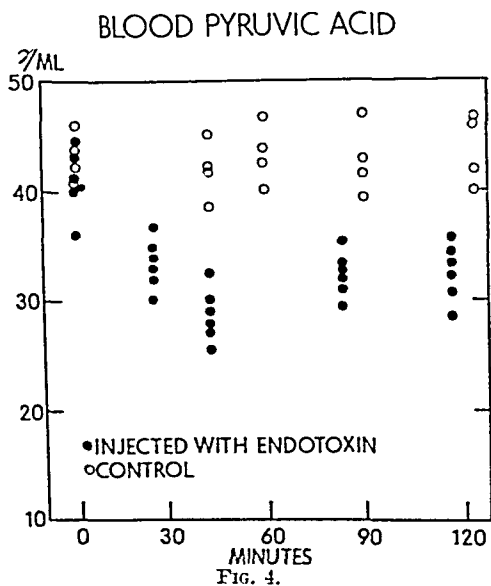


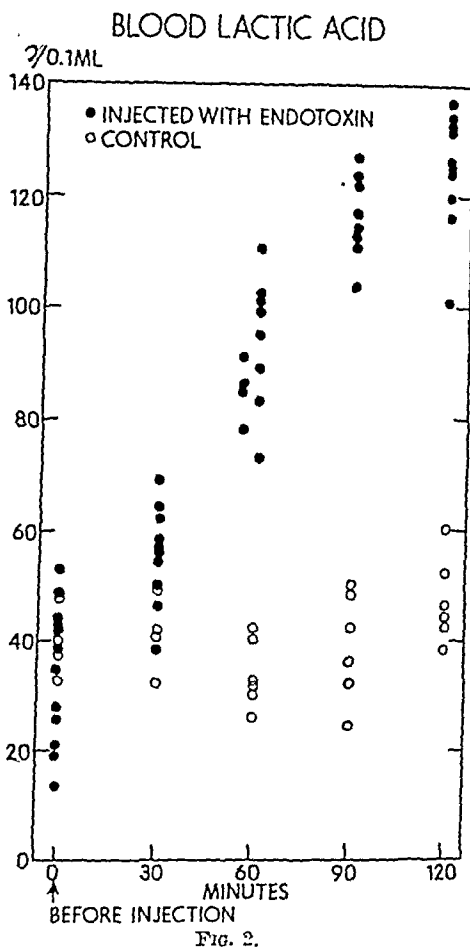
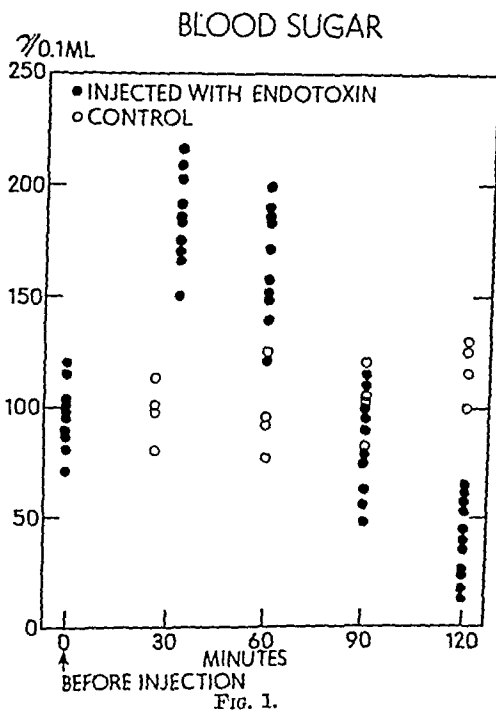
FIG. 4.

¹² Barron, E. S. G., *Advances in Enzymology*, 1943, 3, 149.

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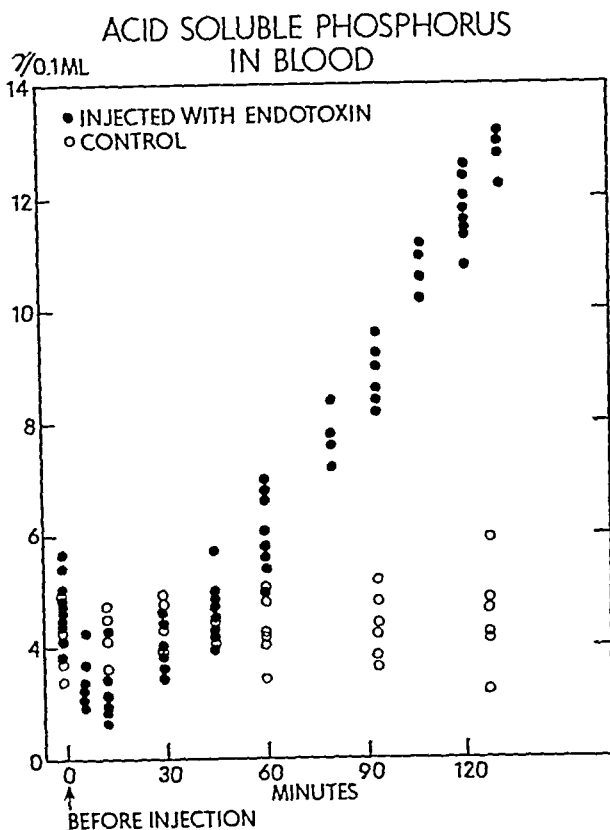


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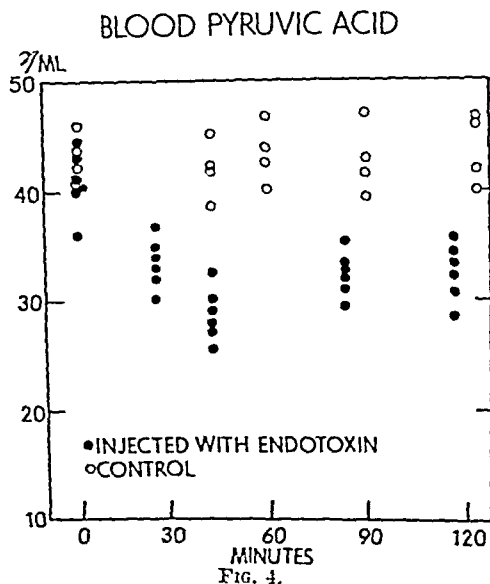


FIG. 4.

¹² Barron, E. S. G., *Advances in Enzymology*, 1943, 3, 149.

EFFECT OF ENDOTOXINS ON CARBOHYDRATE METABOLISM

EFFECT OF ENDOTOXIN ON GLYCOGEN, LACTIC-ACID AND PYRUVIC ACID IN LIVER AND MUSCLE

(IN MILLIGRAMS PER 100 GRAMS DRY WEIGHT OF TISSUES)

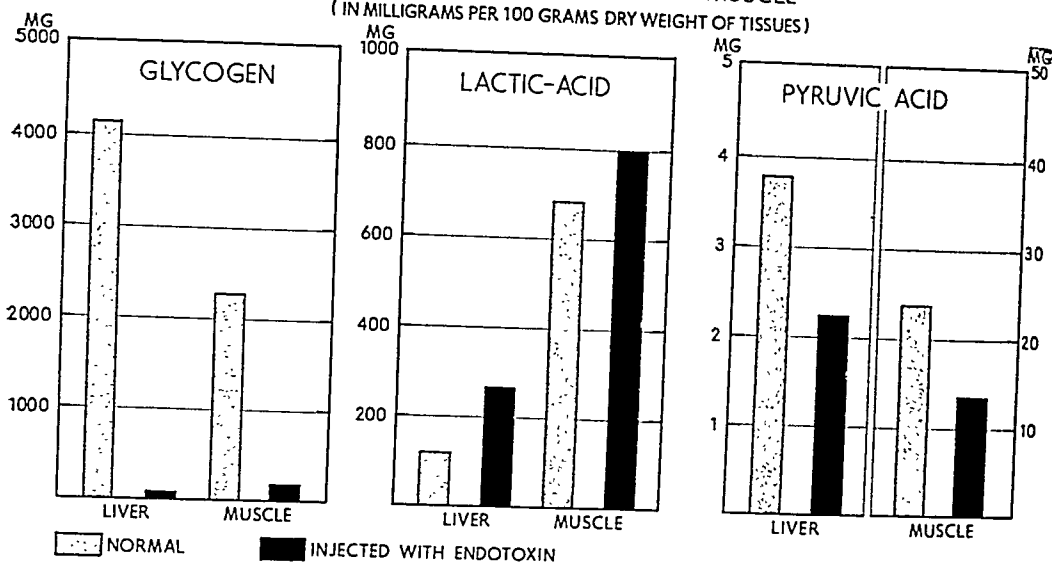


FIG. 5.

TABLE I.

Succinic Dehydrogenase Activity of the Liver and Muscle of Rabbits Injected with Endotoxin.

	Liver		Muscle	
	Normal control	Endotoxin	Normal control	Endotoxin
Meningococcal endotoxin	88.4	28.0	16.2	6.0
	60.1*	40.2*	15.1*	6.5*
	68.9	29.7	24.2	8.4
	80.0	16.0	26.1	8.7
	65.4*	20.4*	19.3*	7.1*
	72.4*	31.0*	29.2*	6.9*
Salmonella endotoxin	62.4	26.1	19.6	5.2
	79.7	15.1	17.1	7.1
	72.4*	20.0*	25.3*	8.0*
	80.1	17.8	22.0	9.2

* These values were obtained from animals which were sacrificed before death from the toxin.

toxins might have produced a state of anoxia in their tissues.

In order to learn more about the mechanism of the tissue anoxia caused by endotoxin, rabbits were injected with meningococcal and *Salmonella* endotoxin[†] as described above and the cytochrome oxidase and succinic-dehydrogenase activity of their muscle and liver were determined by the Warburg technic

as described by Schneider and Potter.¹³ In these enzyme assays phosphate buffer (pH 7.4) was used instead of H₂O for the preparation of tissue homogenates. The importance of this technical detail will be discussed elsewhere. A significant inhibition of succinic dehydrogenase activity was demonstrable. Cytochrome oxidase was not affected (see Table I).

It is interesting to note that the endotoxins

[†] The endotoxin of *Salmonella acrytycke* was prepared in the same way as meningococcal endotoxin.

¹³ Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, 149, 217.

of both *Salmonella* and meningococcus produced similar effects not only in their inhibition of succinic-dehydrogenase but also on the carbohydrate metabolite concentration of tissues. Since the enzyme inhibition occurred in a few minutes after the injection of the endotoxin, it is reasonable to assume that the inhibition of this enzyme activity plays an important role in the mechanism responsible for the changes in carbohydrate metabolite concentration in the poisoned rabbit tissues. The mechanism of this enzyme inhibition and its effect on carbohydrate metabolism is being investigated in detail.

Summary. The intravenous injection of meningococcal or *Salmonella* endotoxin into

rabbits produced increase in blood glucose, lactic acid and inorganic phosphorus. This was followed by hypoglycemia which could be observed before the death of the animal. Liver and muscle glycogen decreased while lactic acid content of the tissues increased. The pyruvic acid content of blood and tissues showed a significant decrease. Succinic dehydrogenase in both muscle and liver was markedly inhibited. Cytochrome oxidase activity was not affected.

The Na-ascorbate used in the enzyme assays was generously supplied by Van Patten Pharmaceutical Company, Chicago.

16260

Passive Cellular Transfer of the Tuberculin Type of Hypersensitivity.*

ABRAM B. STAVITSKY.[†] (Introduced by D. H. Campbell.)

From the Gates and Crellin Laboratories, California Institute of Technology, Pasadena.[‡]

Chase¹ recently reported that the tuberculin type of hypersensitivity was transferred passively to normal guinea pigs by injection of the cells of peritoneal exudate, lymph nodes or spleen of guinea pigs sensitized to tuberculin by the injection of heat-killed tubercle bacilli. The passively conferred sensitivity was transient, lasting about 5 days. This is a significant achievement as the failure passively to transfer tuberculin sensitivity has been adduced as a basic distinction between the tuberculin and the anaphylactic or Arthus types of hypersensitivity.² It was hoped that, if confirmed, the passive cellular transfer of tuberculin hypersensitivity might provide a method suitable for the elucidation

of the underlying chemical factors in the induction and development of this peculiar allergic phenomenon. The repetition of Chase's studies was therefore undertaken.[§]

Materials and Methods. The materials and methods were essentially those used by Chase¹ in his experiments. White albino guinea pigs of both sexes were sensitized to tuberculin by subcutaneous injection into the groin of heat-killed human tubercle bacilli suspended in mineral oil^{3,4,5} and aquafor. Usually 2.5 mg of dried bacillary growth was suspended in a total inoculum of 1 ml for each animal. Five to 9 weeks later, when cutaneous sensitivity to

* Supported by a grant from the Rockefeller Foundation for research in immuno-chemistry.

[†] Present address: Institute of Pathology, Western Reserve University, Cleveland, Ohio.

[‡] Contribution No. 1153.

¹ Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 134.

² Rich, A. R., *The Pathogenesis of Tuberculosis*, Springfield, Charles C. Thomas, 1944.

[§] Since these confirmatory studies have been completed another group of workers reported successful repetition of Chase's experiments. Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Pub. Health Rep.*, 1947, **62**, 994.

³ Freund, J., Casals-Ariet, J., and Hosmer, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 509.

⁴ Freund, J., Casals-Ariet, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67.

⁵ Freund, J., and Gottshall, R. Y., *Arch. Path.*, 1942, **34**, 73.

EFFECT OF ENDOTOXIN ON GLYCOGEN, LACTIC-ACID AND PYRUVIC ACID IN LIVER AND MUSCLE

(IN MILLIGRAMS PER 100 GRAMS DRY WEIGHT OF TISSUES)

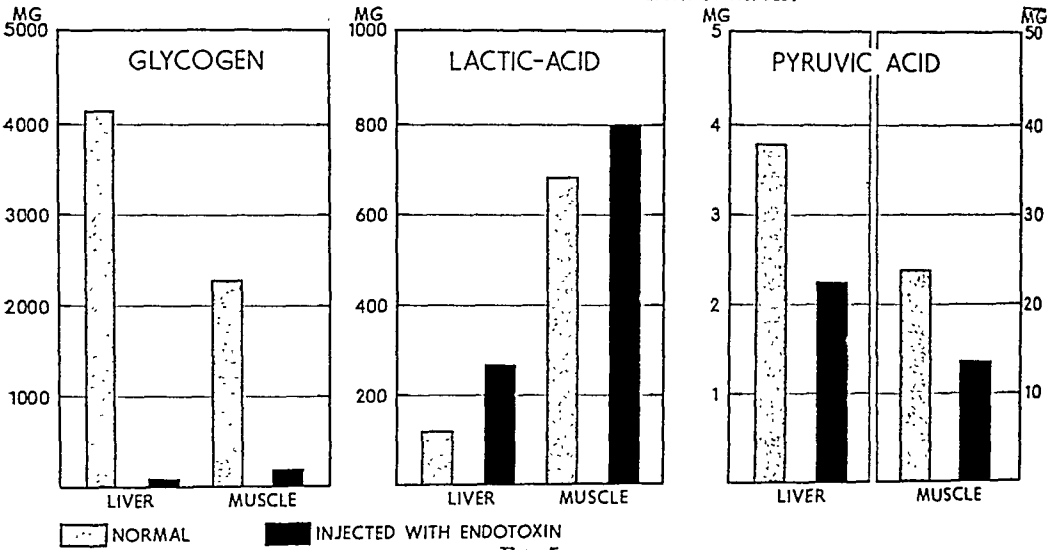


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¹³ Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, 149, 217.

of both *Salmonella* and *meningococcus* produced similar effects not only in their inhibition of succinic-dehydrogenase but also on the carbohydrate metabolite concentration of tissues. Since the enzyme inhibition occurred in a few minutes after the injection of the endotoxin, it is reasonable to assume that the inhibition of this enzyme activity plays an important role in the mechanism responsible for the changes in carbohydrate metabolite concentration in the poisoned rabbit tissues. The mechanism of this enzyme inhibition and its effect on carbohydrate metabolism is being investigated in detail.

Summary. The intravenous injection of meningococcal or *Salmonella* endotoxin into

rabbits produced increase in blood glucose, lactic acid and inorganic phosphorus. This was followed by hypoglycemia which could be observed before the death of the animal. Liver and muscle glycogen decreased while lactic acid content of the tissues increased. The pyruvic acid content of blood and tissues showed a significant decrease. Succinic dehydrogenase in both muscle and liver was markedly inhibited. Cytochrome oxidase activity was not affected.

The Na-ascorbate used in the enzyme assays was generously supplied by Van Patten Pharmaceutical Company, Chicago.

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Passive Cellular Transfer of the Tuberculin Type of Hypersensitivity.*

ABRAM B. STAVITSKY.[†] (Introduced by D. H. Campbell.)

From the Gates and Crellin Laboratories, California Institute of Technology, Pasadena.[‡]

Chase¹ recently reported that the tuberculin type of hypersensitivity was transferred passively to normal guinea pigs by injection of the cells of peritoneal exudate, lymph nodes or spleen of guinea pigs sensitized to tuberculin by the injection of heat-killed tubercle bacilli. The passively conferred sensitivity was transient, lasting about 5 days. This is a significant achievement as the failure passively to transfer tuberculin sensitivity has been adduced as a basic distinction between the tuberculin and the anaphylactic or Arthus types of hypersensitivity.² It was hoped that, if confirmed, the passive cellular transfer of tuberculin hypersensitivity might provide a method suitable for the elucidation

of the underlying chemical factors in the induction and development of this peculiar allergic phenomenon. The repetition of Chase's studies was therefore undertaken.[§]

Materials and Methods. The materials and methods were essentially those used by Chase¹ in his experiments. White albino guinea pigs of both sexes were sensitized to tuberculin by subcutaneous injection into the groin of heat-killed human tubercle bacilli suspended in mineral oil^{3,4,5} and aquafor. Usually 2.5 mg of dried bacillary growth was suspended in a total inoculum of 1 ml for each animal. Five to 9 weeks later, when cutaneous sensitivity to

* Supported by a grant from the Rockefeller Foundation for research in immuno-chemistry.

[†] Present address: Institute of Pathology, Western Reserve University, Cleveland, Ohio.

[‡] Contribution No. 1153.

¹ Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **50**, 134.

² Rich, A. R., *The Pathogenesis of Tuberculosis*, Springfield, Charles C. Thomas, 1944.

[§] Since these confirmatory studies have been completed another group of workers reported successful repetition of Chase's experiments. Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Pub. Health Rep.*, 1947, **62**, 994.

³ Freund, J., Casals-Ariet, J., and Hosmer, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 509.

⁴ Freund, J., Casals-Ariet, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67.

⁵ Freund, J., and Gottshall, R. Y., *Arch. Path.*, 1942, **34**, 73.

EFFECT OF ENDOTOXIN ON GLYCOGEN, LACTIC-ACID AND PYRUVIC ACID IN LIVER AND MUSCLE

(IN MILLIGRAMS PER 100 GRAMS DRY WEIGHT OF TISSUES)

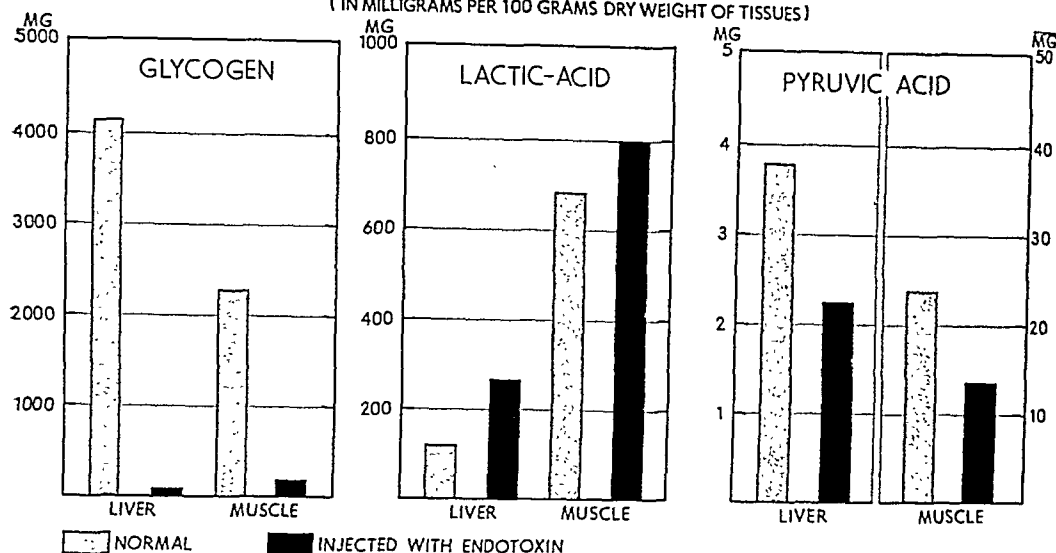


FIG. 5.

TABLE I.

Succinic Dehydrogenase Activity of the Liver and Muscle of Rabbits Injected with Endotoxin.

	Liver		Muscle	
	Normal control	Endotoxin	Normal control	Endotoxin
Meningococcal endotoxin	88.4	28.0	16.2	6.0
	60.1*	40.2*	15.1*	6.5*
	68.9	29.7	24.2	8.4
	80.0	16.0	26.1	8.7
	65.4*	20.4*	19.3*	7.1*
	72.4*	31.0*	29.2*	6.9*
Salmonella endotoxin	62.4	26.1	19.6	5.2
	79.7	15.1	17.1	7.1
	72.4*	20.0*	25.3*	8.0*
	80.1	17.8	22.0	9.2

* These values were obtained from animals which were sacrificed before death from the toxin.

toxins might have produced a state of anoxia in their tissues.

In order to learn more about the mechanism of the tissue anoxia caused by endotoxin, rabbits were injected with meningococcal and *Salmonella* endotoxin† as described above and the cytochrome oxidase and succinic-dehydrogenase activity of their muscle and liver were determined by the Warburg technic

as described by Schneider and Potter.¹³ In these enzyme assays phosphate buffer (pH 7.4) was used instead of H₂O for the preparation of tissue homogenates. The importance of this technical detail will be discussed elsewhere. A significant inhibition of succinic dehydrogenase activity was demonstrable. Cytochrome oxidase was not affected (see Table I).

It is interesting to note that the endotoxins

† The endotoxin of *Salmonella aertrycke* was prepared in the same way as meningococcal endotoxin.

¹³ Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, 149, 217.

In general the data confirm Chase's results on the passive transfer of tuberculin hypersensitivity with the further observation that whole blood may occasionally transfer sensitivity. In a few instances by means of daily skin tests it was found that the passively conferred sensitivity lasted only 4 or 5 days. The occasional failures in transfer of sensitivity with peritoneal exudate or lymph node cells have not been satisfactorily explained.

Discussion. It should be emphasized that the positive tuberculin reactions elicited in the recipient animals were of the delayed type typical of reactions in animals actively sensitized by injection of tubercle bacilli.² The reactions rarely appeared much before 24 hours and were at their height of intensity 48 to 72 hours after injection.

It is interesting to note that sensitivity was transferred in 2 instances by injection of whole blood from sensitized animals. This is not surprising as Chase has found recently⁶ that the buffy coat separated from the red cells of the blood may passively transfer sensitivity. These observations and the demonstration of typhoid agglutinins in the buffy coat from the blood of immunized rabbits⁷ suggest a wider than hitherto supposed participation of the white cells of the blood in immunological phenomena.

The positive results employing suspensions containing about 95% lymphocytes are indicative of the possible preeminence of this cell in the transfer of sensitivity. The specific lysis of a portion of small mature lymphocytes from blood or spleen of tuberculous mice and guinea pigs upon contact with tuberculin⁸ may be related to the ability of lymphocytes to transfer sensitivity. The recent implication of lymphocytes as cells which synthesize antibodies⁹ may or may not be a related finding, as it has not been shown so far that

antibodies are involved in the development of tuberculin hypersensitivity.² The demonstration¹⁰ that cells from tuberculin-sensitive animals may retain their sensitivity to the toxic action of tuberculin after several transplants in tissue culture would seem to rule out the role of circulating antibodies in this phenomenon.

Work is proceeding in the attempt to isolate the factor responsible for the transfer of sensitivity. Thus far efforts to transfer sensitivity by means of various crude extracts of the cells have been completely negative. In the endeavor to determine whether the cell transfer is a general phenomenon, cell transfer of the delayed type of pneumococcus sensitivity in rabbits¹¹ has been tried. Thus far these experiments also have proven unsuccessful.

Summary. 1. In confirmation of Chase's studies, the tuberculin type of hypersensitivity has been transferred passively to normal, unsensitized guinea pigs by injection of the cells of peritoneal exudate, lymph nodes, spleen, and whole blood of tuberculin-sensitive guinea pigs. The passively conferred sensitivity lasted 4 or 5 days.

2. The transfer of sensitivity by injection of whole blood taken with other observations suggests that the white cells of the circulating blood may participate more widely than previously believed in immunological phenomena.

3. The positive results employing suspensions containing 95% of lymphocytes suggest that this cell type may be of great importance in the transfer of sensitivity.

The author is grateful to Dr. Dan H. Campbell for interest and encouragement in the course of these studies.

⁹ Ehrlich, W. E., and Harris, T. N., *Science*, 1945, **101**, 28.

¹⁰ Moen, J. K., and Swift, H. K., *J. Exp. Med.*, 1936, **64**, 339.

¹¹ Julianelle, L. A., *J. Exp. Med.*, 1930, **51**, 463.

⁷ Stavitsky, A. B., unpublished observations.

⁸ Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.

TABLE I.
Reactions to Tuberculin in Guinea Pigs Injected with Cells from Normal and Tuberculin-sensitized Guinea Pigs.

No. and type donor animals	Volume and type material transferred to each recipient*	No. of recipients*	Avg reaction (mm) of recipients		No. tuberculin pos.†
			24 hr after injection of tuberculin‡	and 48 hr	
15 sensitized	0.5 ml peritoneal cells	3	8 × 9, i, e	12 × 13, i, e	3/3
16 "	0.65 " " "	4	8 × 8, i, e	10 × 12, i, e	3/4
4 "	— " " "	1	8 × 8, e	12 × 15, i, e	1/1
2 "	— " " "	1	7 × 7, i	10 × 10, i, e	1/1
9 "	2.0 ml splenic cells	3	10 × 9, i, e	12 × 12, i, e	3/3
9 "	2.0 " lymph node cells	3	10 × 10, i, e	12 × 12, i, e	2/3
9 "	19.0 " defibrinated blood	3	9 × 9, i, e	10 × 11, i, e	1/3
9 "	20.0 " citrated blood	3	8 × 8, i, e	11 × 11, i, e	1/3
12 "	8.0 " serum	4	0	0	0/4
16 normal	0.70 " peritoneal cells	4	0	0	0/4
16 "	2.25 " splenic cells	4	0	0	0/4

* For example, in the first group peritoneal cells from 15 sensitized animals were divided into 3 parts and injected into each of 3 animals; in second group, cells from 16 animals were divided into 4 parts, and so on.

† Reactions to second injection of tuberculin 48 hours after passive transfer. i = induration; e = erythema; 0 = negative.

‡ In first group, for example, 3 out of 3 animals tested reacted positively to tuberculin; in second group 3 out of 4, and so on.

tuberculin was found to be definite and intense, exudates were evoked in the sensitized guinea pigs by the intraperitoneal inoculation of 30 ml of mineral oil. Forty-eight hours later the peritoneal cavities were flushed out with citrated-Locke's solution containing 0.25% gelatin. The washings were placed in a separatory funnel and allowed to stand. After the oil had risen the aqueous layer was collected and centrifuged lightly for several minutes. The sedimented cells, about 0.15 ml from each animal, were resuspended in citrated gelatin-Locke's solution and again centrifuged. The washed cells were suspended in about 4 ml of gelatin-Locke's solution and immediately inoculated intraperitoneally into male albino guinea pigs. The differential cell count of the suspension before inoculation revealed about 25% polymorphonuclear leukocytes, 35% lymphocytes, and 40% mononuclears.

Twenty-four and 48 hours after the cell-transfer the recipient animals were tested for tuberculin sensitivity by intradermal injection of 1:2 or 1:4 Old Tuberculin from which the glycerin had been removed by precipitation with cold alcohol.⁶

As a control each animal was also skin-

tested with 0.1 ml of 1:2 or 1:4 glycerin broth which was deglycerinated in the same manner as was the Old Tuberculin. It had been previously determined that these concentrations of "deglycerinated" tuberculin and broth were the highest not producing visible reactions upon injection into the skin of normal, unsensitized guinea pigs. The cutaneous response to all test inoculations was measured 24 and 48 hours after inoculation of the test material.

Cells from the spleen and lymph nodes and, in a few instances, whole defibrinated or citrated blood and serum from sensitized animals also were employed in the attempt to transfer sensitivity. It is important to note that smears of the lymph node cell suspensions revealed that approximately 95% of the cells were small or medium-sized lymphocytes and 5% monocytes. As controls corresponding types of cells from normal, unsensitized guinea pigs were inoculated into normal guinea pigs.

Results. Pertinent data and results of these experiments are summarized in Table I. In order to conserve space the uniformly negative results of control injections of "deglycerinated" broth and of all tests 24 hours after the cell transfer are omitted from the table.

⁶ Chase, M. W., personal communication.

In general the data confirm Chase's results on the passive transfer of tuberculin hypersensitivity with the further observation that whole blood may occasionally transfer sensitivity. In a few instances by means of daily skin tests it was found that the passively conferred sensitivity lasted only 4 or 5 days. The occasional failures in transfer of sensitivity with peritoneal exudate or lymph node cells have not been satisfactorily explained.

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⁹ Ehrlich, W. E., and Harris, T. N., *Science*, 1945, **101**, 28.

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¹¹ Julianelle, L. A., *J. Exp. Med.*, 1930, **51**, 463.

⁷ Stavitsky, A. B., unpublished observations.

⁸ Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.

'Accelerator Globulin' and 'Antihemophilic Globulin' in Thrombin Formation from Aged Prothrombin and in Hemophilic Blood.*

JOHN H. FERGUSON AND JESSICA H. LEWIS.†

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Recent studies¹⁻⁹ have shown that a newly recognized factor is important in the conversion of prothrombin to thrombin. The plasma "accelerator globulin" of Ware, Guest, and Seegers,^{3,4,9} used in the present studies, contains this factor and is, in all probability, similar to Quick's "labile factor"⁶ and Owren's "factor V."¹ "Antihemophilic globulin"¹⁰ is a plasma protein fraction which restores the "thromboplastic" defect and thus accelerates the thrombin formation in hemophilic blood. The following experimental data were obtained during current *in vitro* studies of the various factors involved in the process of thrombin formation from purified prothrombin preparations.

* This is the sixth of a series of studies aided by a grant from the John and Mary R. Markle Foundation.

† Postdoctorate Fellow, U. S. Public Health Service.

1 Owren, P. A., *The Coagulation of Blood. Investigations on a New Clotting Factor*, Oslo, 1947.

2 Fantl, P., and Nance, M., *Nature*, London, 1946, **158**, 708.

3 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

4 Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

5 Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

6 Quick, A. J., *Am. J. Physiol.*, 1947, **151**, 63.

7 Honorato, R., *Am. J. Physiol.*, 1947, **150**, 381, 405.

8 Munro, M. P., and Munro, F. L., *Am. J. Physiol.*, 1947, **150**, 409.

9 Ware, A. G., Murphy, R. C., and Seegers, W. H., *Science*, 1947, **106**, 618.

10 Lewis, J. H., Tagnon, H. J., Davidson, C. S., Minot, G. R., and Taylor, F. H. L., *Blood* (the *J. Hematol.*), 1946, **1**, 166.

11 Ferguson, J. H., *Blood* (the *J. Hematol.*), vol. honoring G. R. Minot, in press.

12 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 285, 302.

Reagents (cf. ^{11,12}). 1. *Borate buffer* (buff.), pH 7.7, solvent and diluent throughout. 2. *Prothrombin* (PRO.) from bovine plasma, courtesy of Dr. W. H. Seegers and colleagues (Wayne Univ.) PRO. G partially purified (product 5 of reported procedures)¹³ potency 720 "units" per mg (dry wt). PRO. H, highly purified (ammonium sulfate fractionation of product 8),¹³ potency 990 "units" per mg (dry wt = 10 × tyrosine). 3. *Thromboplastin* (tpln.) 0.2% rabbit lung preparation (Squibb's). 4. *CaCl₂* (Ca) M/20. 5. *Fibrinogen* (B.F.), 0.5% Armour's bovine plasma Fraction I (55% "clottable"). 6. "Antihemophilic globulin" (HF), Harvard¹⁴ human Fraction I, low in fibrinogen (HF₁ 37%; HF₂ 18%). 7. "Accelerator globulin" (AcG), from bovine plasma (Seegers *et al.*), contained a small amount of prothrombin.

Methods. 0.1% solutions of PRO., AcG, and HF were divided into 2 portions, part being freshly frozen and kept at -20°C until tested and part being aged at 28°C (thermostat water bath) for 7 days. 5 cc (total vol.) of thrombic mixtures (T), in borate buffer, contained 0.1% PRO., to dilution noted in Tables I, II, 0.5 cc Ca, 0.5 cc tpln (approximately optimal), and 0.5 cc of the various agents noted. At stated incubation periods (i.t.), 0.25 cc T was added to 0.5 cc BF, at 28°C, and clotting-time (c.t.) noted.

"Antihemophilic" activity was measured by addition of 2 cc of fresh hemophilic blood to 0.1 cc of test preparations and measurement of the coagulation time at 37°C.

Results. In the data of Table I, the prothrombin (PRO H) after aging 1 week at 28°C, when diluted and activated with the stated amounts of calcium and thrombo-

¹³ Seegers, W. H., Loomis, E. C., and Vandenberg, J. M., *Arch. Biochem.*, 1945, **6**, 85.

¹⁴ Edsall, J. T., *Adv. Prot. Chem.*, 1943, **3**, 383.

TABLE I.

Effects of "Accelerator Globulin" (AcG) and "Antihemophilic Globulin" (HF) on Activation of *Aged* and *Frozen* Prothrombin (Pro.).

T = thrombic mixtures, containing (final concentrations) Pro.H (1:20,000), tpln. (1:5000), Ca (M/200), and agents cited (1:10,000), with borate buffer (pH = 7.7) to 5 cc vol. Clotting times (c.t.), sec., at 28°C, for 0.25 cc T + 0.5 cc BF (0.5%), at stated incubation periods (i.t.).

T.	Pro.	Agent	Incubation time					
			5m	10m	15m	20m	30m	1 hr
1.	Aged	—	190	129	105	72	50	30
2.	"	AcG (aged)	137	104	78	61	43	23
3.	"	" (froz.)	17	11	10	10	9½	8
4.	"	HF ₁ (aged)	165	107	101	98	75	47
5.	"	" (froz.)	161	122	106	100	91	58
Clotting time (sec.)								
6.	Froz.	—	16	10	8½	8	8	8
7.	"	AcG (aged)	12	9½	8	8	8	8
8.	"	" (froz.)	12	8	8	8	8	8
9.	"	HF ₁ (aged)	26	16½	13	12½	12	12
10.	"	" (froz.)	29	19	15	13	13	12½
11.	—	AcG (froz.)	120	95	73	71	62	53
12.	—	" (aged)	220	220	225	220	240	300

plastin, showed very slow activation (T_1) which was incomplete even after 4 hours incubation. *Aged* AcG exerted only a negligible improvement (T_2) as compared with *frozen* AcG (T_3) which was able, in less than an hour, to restore the full thrombic potency as shown by the 8" c.t. identical with that reached in T_6 , T_7 , T_8 . The prothrombin kept in the *frozen* state, when activated in the usual manner with Ca and tpln. (T_6) completed the thrombin formation in less than 20 min., and showed only slight acceleration by *aged* AcG (T_7) and still minor improvement (10 min. i.t.) by *frozen* AcG (T_8). HF₁ did not show any accelerator effects with either prothrombin, but, on the contrary, evinced some definite interference with thrombin formation, though somewhat less with *aged* HF (T_4 , T_9) than with *frozen* HF (T_5 , T_{10}). Small amounts of fine flocculant fibrin which appeared during the incubation of these mixtures did not interfere with the tests and were not considered significant.

Controls. The fibrinogen (BF) contained a mere trace of prothrombin which caused negligible fibrin shreds to appear in about 25 minutes when tested with the same amounts of Ca and tpln. (in buff.) as used in the other tests. HF controls, similarly activated, barely increased these traces of fibrin, now appearing in about 20 minutes. The AcG

preparation, as expected, did show a little content of prothrombin estimated at less than 10%, in the *frozen* solution (T_{11}). The *aged* AcG showed no prothrombin activity but did contain a trace of active thrombin which deteriorated somewhat as the incubation proceeded (T_{12}). It is not conceivable that these very minor sources of prothrombin and thrombin could be at all significant in explaining the "accelerator" effect noted in T_3 .

Similar experiments with a less purified prothrombin (PRO. G) gave exactly the same results. In Table II, A the data are presented as "percentages" of thrombin yield, derived from the clotting-times (Table II, B) obtained by a series of dilutions of T_2 at maximal activity (4 hrs). T_1 : the *aged* PRO., diluted and activated in the usual manner with optimal Ca and tpln., is only about one-third activated in 4 hrs but is estimated as 80% complete in 3 days. T_2 : the addition of 1 cc *fresh* (frozen) 1:1000 AcG gave over 90% of maximal thrombin formation in 10 min. and 100% (complete activation) in less than 4 hours. T_3 : HF₂, another "antihemophilic globulin" preparation, one-tenth vol. (0.5 cc) of *fresh* 1:1000 soln., showed the same kind of retarding action previously noted with HF₁ (Table I), the best value for thrombin yield (i.e. 2-3 days) not exceeding 20%.

Although PRO. G was elsewhere¹¹ shown

TABLE II.

A. Thrombin Yield, "percentage" activation, after varying periods of incubation (i.t.), in mixtures (T) containing PRO.G. (1:12,500) aged 7 days at 28°C, tpln. (1:5000), Ca (M/200), and added agents (mg per cc) noted. 0.25 cc T + 0.5 cc BF. 28°C. pH = 7.7.

T.	Agent	5 min	10 min	1 hr	4 hr	1d	2d	3d (i.t.)	Clot lysis (37°)
1.	buff.	tr.	tr.	17	34	50	75	80	None (5 days)
2.	AcG (0.2)	44	93	95	100	100	96	80	" "
3.	HF ₂ (0.1)	tr.	tr.	3	6	15	20	20	" "

B. "Percentage" Dilutions of T ₂ (4 hr incub. time): clotting-times (sec.).								
Dil.	100%	80%	60%	40%	20%	10%		5%
Coag. time (sec)	10	15	18	21½	37	46½		89

TABLE III.

"Antihemophilic" Activity.

2 cc freshly drawn hemophilic blood added to 0.1 cc 0.1% solutions of agents cited. Coagulation times (C.T.), min., at 37°C.

Agent:	Saline (control)	AcG (froz.)	AcG (aged)	HF (froz.)	HF (aged)
Coag. time (min.)	142	98	98	27	60

to contain a trace of protease contaminant, the clot-lysis tests recorded in Table II, A show no appreciable enzyme activity in the dilution used.

In other (not cited) experiments with PRO. G solution freshly prepared and activated (a) with and (b) without fresh (frozen) AcG, a maximal thrombic potency represented by c.t. of 8 sec. was reached in (a) 20 min. or (b) 30 min., respectively. This confirms the evidence in Table I (T₂, T₇) showing that these *fresh* or frozen prothrombin preparations contain an abundance of the AcG factor. In earlier experiments,¹¹ testing stronger (0.2-0.4%) solutions of this same prothrombin, identical activation data (with optimal Ca and tpln.) were obtained with fresh solutions as compared with the same solutions after 3-4 weeks storage at ice-box temperature (5°C).

Table III shows the antihemophilic activity of AcG and HF₁. Even in the high (<0.005% final) dilution, frozen HF₁ showed marked acceleration of clotting. This activity was greatly diminished by aging. AcG both frozen and aged showed only minimal effects.

Discussion. The data clearly show that the major change in "aged" prothrombin is an impaired ability to form thrombin with the classical activators, namely, calcium and thromboplastin. Fresh (or frozen) "accelerator globulin" greatly speeds up the conver-

sion and restores the thrombin yield (cf. 7). in fact completely in T₃ (Table I), but "aging" of the AcG causes it to lose this property. Fresh (or frozen) prothrombin evidently contains an adequacy of this factor, as a contaminant⁶ but it deteriorates on aging at or above room temperature. This deterioration is emphasized by dilution of the prothrombin preparation. There is little doubt that the validity of estimations of the thrombin-yielding potency of prothrombin depends upon adequate control of the "accelerator" factor.⁸ Our findings in regard to highly purified (Seegers) prothrombin are comparable to the data of other workers investigating the thrombin-yield changes in stored and otherwise treated plasma. Interpretation in terms of presence or instability of AcG possibly explains most of the divergence of viewpoints noted in the literature and accounts for the prothrombin preserving and activity restoring powers credited to plasma¹⁵ or certain fractions,^{1,3,15} including some fibrinogens.^{7,8,16}

Questions of possible deterioration of prothrombin proper or of thrombin in highly dilute solutions¹¹ are raised by the later data of Table II, but the analysis of these prob-

¹⁵ Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 68.

¹⁶ Loomis, E. C., and Seegers, W. H., *Am. J. Physiol.*, 1947, **148**, 563.

lems is beyond the scope of the present inquiry. The same is true of the explanation for the evident interference in thrombin formation by our weak HF solutions and the only conclusion, for the present, is that "antihemophilic globulin" lacks the accelerator effect.

We have shown elsewhere¹² that HF contains a protease precursor which, when activated by streptokinase, evinces proteolytic (e.g., fibrinogenolytic, etc.) and "thromboplastic" (aiding thrombin formation) properties, similar to other plasma protease preparations. The negative clot-lysis tests included in Table II, A, show that, for reasons of dilution and lack of activation such protease plays no part in the present results.

AcG has been studied in several types of experiment with completely negative results as regards any indication that it may be related to the plasma protease¹⁷ system, whether as active enzyme or precursor, or as activator or inhibitor of added precursor, streptokinase, or active protease. It is an important point, therefore, that the aid which the "accelerator globulin" affords to thrombin formation has no demonstrable relation to the plasma protease system (*cf.* 1).

It is also of interest to note that AcG has no significant "antihemophilic" activity,

¹⁷ Ferguson, J. H., *Science*, 1943, **97**, 319; 1947, **105**, 488.

which, of course, is to be expected from the normal prothrombin clotting times obtained in hemophilia, indicating that hemophilic blood has adequate amounts both of prothrombin and the accelerator factor.

Conclusions. 1. There is a new factor, or group of factors, (Quick's "labile factor," Owren's "factor V," Ware, Guest and Seegers' "accelerator globulin"), to be recognized in the first phase of the blood-clotting system. At present it may be considered an "accessory" activator distinct from calcium, thromboplastin, and thromboplastic enzyme. 2. This factor is found abundantly in fresh plasma and prothrombin (purified by the usual methods, *e. g.* ¹³), but deteriorates on "aging," independently of the prothrombin. High dilution may be necessary to demonstrate this. The addition of fresh AcG will restore the original potency of the prothrombin, but aged AcG loses this property. 3. AcG is not "antihemophilic" and so-called "antihemophilic globulin" is not able to restore the activity of aged prothrombin. 4. "Owren's disease,"¹⁸ a specific hemorrhagic disorder associated with a plasma deficiency of this new factor, is, in all probability, a true clinical entity, which suitable tests can differentiate from "idiopathic hypoprothrombinemia," hemophilia, and other bleeding diseases.

¹⁸ Owren, P. A., *Lancet*, 1947, **252**, 446.

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Prothrombin Conversion Factor of Dicumarol Plasma.*

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Dicumarol *in vivo* is generally believed to cause a deficiency of plasma prothrombin. The therapeutic value of serum^{1,2} in the treat-

ment of cattle with "hemorrhagic sweet clover disease," a dicumarol-induced diathesis is therefore puzzling.

An investigation was conducted on dogs to which dicumarol was administered orally or

* Abridgment of a portion of the thesis submitted by Dr. Owen to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D. in Medicine.

¹ Schofield, F. W., *J. Am. Vet. M. A.*, 1924, **64**, 553.

² Roderick, L. M., *Am. J. Physiol.*, 1931, **96**, 413.

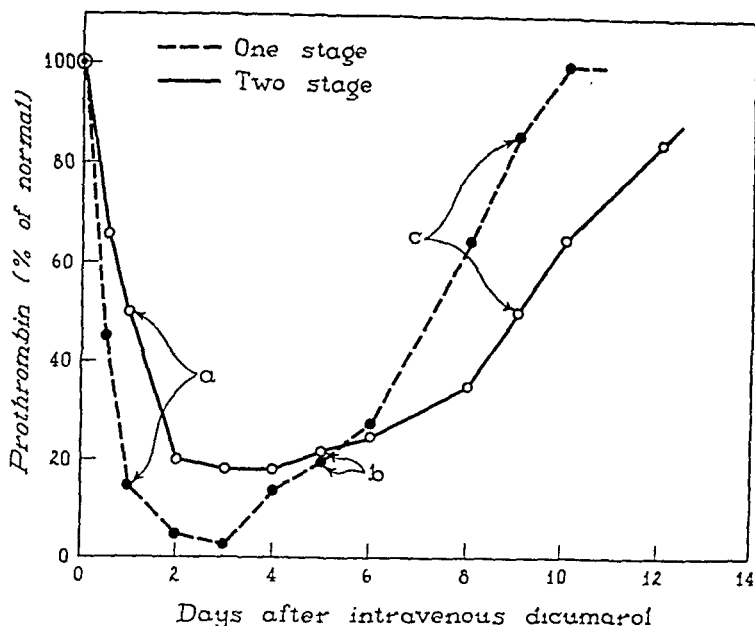


FIG. 1.

Changes of the prothrombin level as determined by the one-stage³ and the 2-stage methods after intravenous administration of dicumarol. For explanation of a, b, and c see text.

intravenously. Prothrombin determinations were performed by the one-stage test of Quick³ and the 2-stage test of Warner, Brinkhous, and Smith.⁴

To increase the sensitivity of the Quick test, plasmas were studied at a number of dilutions, 0.3% fibrinogen being used as diluent; percentages were interpolated from the dilution curves of control samples.

In the 2-stage test, plasma is freed of fibrinogen by the addition of weak thrombin, which in turn is rapidly inactivated. The defibrinated plasma is diluted; calcium and thromboplastin are added and portions of the solution are mixed with fibrinogen, the clotting times being noted. Under standard conditions a coagulation time of 15 seconds denotes 1 unit of thrombin per milliliter, which is presumed to have arisen from 1 unit of prothrombin per milliliter. Since normal dog plasma, diluted about 300-fold, yields 1

unit of thrombin per milliliter, the undiluted plasma is said to contain about 300 units of prothrombin per milliliter. By determination of the concentration of thrombin at frequent intervals during its evolution not only the amount of thrombin formed but also the time for its formation are measurable.

As expected, the prothrombin level dropped after dicumarolization—the extent of the fall and its duration varying with the dose.⁵ Fig. 1 shows the characteristic course in a female dog, weighing 8.5 kg, given 10 mg of dicumarol per kilo of body weight, by the intravenous route.

According to the Quick test the prothrombin fell more rapidly, to lower levels, but returned to normal sooner than by the 2-stage test. Thus, a sample of blood taken 24 hours after the drug had been administered (a) showed 50% prothrombin by the 2-stage test and 15% by the one-stage test. On the fifth day (b) the two methods were in close agreement. However, on the ninth day (c), with

³ Quick, A. J., Stanley-Brown, Margaret, and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501.

⁴ Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

⁵ Bollman, J. L., and Preston, F. W., *J. A. M. A.*, 1942, **120**, 1021.

the level determined by the 2-stage test at 50% again, the prothrombin as determined by Quick's method had returned to almost normal levels.

Repetition of this experiment invariably yielded similar results. If smaller doses of dicumarol were employed, a point was reached (1 mg per kilo of body weight) at which the prothrombin, as measured by the 2-stage test, fell very little (to not less than 80%) while by the Quick test it dropped to a 40 or 50% level.

Study of the rate of conversion of prothrombin to thrombin seemed to indicate the cause of the discrepancy between the two methods. "Conversion times" represent the time required for conversion of 80% of the prothrombin into thrombin after addition of calcium and thromboplastin to plasma diluted to a prothrombin concentration of 1 unit per milliliter. It was found that when the Quick test yielded lower results than the 2-stage test, conversion of the prothrombin was delayed; when the tests agreed, conversion rates were normal; or, when the one-stage results were higher, conversion rates were accelerated above normal. Thus, in Fig. 1, at the predicumarol level (100%) conversion of 1 unit of prothrombin was 80% complete in 30 seconds;⁶ at (a) in 80 seconds; at (b) in 30 seconds; at (c) in 20 seconds.

In an attempt to discover the cause of the variable conversion rates of prothrombin it was noted that mixture of as little as one part of normal plasma with nine parts of slowly converting "dicumarol plasma" completely corrected the hypoconvertibility (from 80 seconds to 30 seconds) and likewise closed the gap between the one-stage and 2-stage tests. For example, at point (a) in Fig. 1, when 9 parts of the "dicumarol plasma" were mixed with one part of control plasma, the 2-stage test now read 52% (theoretically 55%); a Quick test on this mixture yielded 55% instead of the expected 24%. Further, addition of normal serum, free of prothrombin, thrombin, and fibrinogen, was equally corrective. Thus, some factor would seem to be present in normal serum, as well as in plasma, which can accelerate delayed convertibility.

Ammonium sulfate fractionation of normal dog serum revealed traces of the "conversion factor" in 25% and 33% saturation fractions, with the highest concentration at about 40 or 50% saturation. Dilution of serum and acidification to pH 5.3, followed by exposure to magnesium hydroxide, revealed little adsorption unless a great excess of the insoluble hydroxide was used. The factor, like prothrombin, is associated with the pseudoglobulins, but differs from prothrombin by being poorly adsorbed by magnesium hydroxide. Its deterioration at extremes of pH (5.5, 10.0), with moderate heating (50°C), and on exposure to air is much greater than that of prothrombin.

An analysis of dog plasma kept at 4°C for several weeks confirmed the findings reported in human and ox plasma;⁶ that is, a rapid fall of prothrombin as measured by Quick's method, a slower fall as measured by the 2-stage test. Study of the rate of conversion of prothrombin of dog plasma kept at 4°C was made at intervals for a period of more than 60 days. Progressive hypoconvertibility was found to be the cause of the discrepancy between the methods. Ware, Guest and Seegers⁶ have attributed the disparity to deterioration of fibrinogen. However, their data show incomplete correction when the fibrinogen of the plasma was replenished. While the fibrinogen must be adequate for performance of the Quick test, we find that correction of delayed conversion and of the difference between the prothrombin methods is possible only on addition of the conversion factor—such as in plasma, serum, or their pseudoglobulins.

There is a close resemblance between this conversion factor and Owren's factor V,⁷ present in Seitz-filtered plasma, or the activators of Fantl and Nance⁸ and of Ware, Guest, and Seegers,⁶ as well as Quick's "pro-

⁶ Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 58.

⁷ Owren, P. A., *Acta med. Scandinav.*, 1947 (suppl.), **194**, 327 pp.

⁸ Fantl, P., and Nance, M., *Nature*, London, 1946, **158**, 708.

⁹ Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

thrombin A."¹⁰ The presence of the factor in serum might explain Schofield's¹ and Roderrick's² therapeutic findings—findings difficult to understand if deterioration of prothrombin alone were present in cases of dicumarol or toxic sweet clover poisoning.

Summary. Evidence is presented that suggests the disappearance not only of prothrombin in dogs treated with dicumarol, but also of a factor important in the conversion of

prothrombin to thrombin. Like prothrombin, this factor is associated with the plasma pseudoglobulins, but unlike prothrombin it is plentiful in serum. Addition of plasma, serum or serum pseudoglobulin corrects the delayed prothrombin convertibility of early dicumarolization or of stored plasma. The discrepancy between the one-stage and 2-stage methods of estimating prothrombin seems to reside in this conversion factor.

¹⁰ Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

16263

Immunological Studies of Newcastle Virus.

REGINALD L. REAGAN, MARY G. LILLIE, JEAN E. HAUSER, AND A. L. BRUECKNER.

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A California strain of Newcastle virus, No. 11,914, which was adapted to the Syrian hamster,¹ proved valuable as an immunizing agent in chickens against the virulent unmodified virus of this strain.² This successful immunization prompted continued experiments to study the relationship of the living modified virus as an immunizing agent, not only against the California strain but also against a Colorado strain, No. 8518, and a Connecticut strain, secured from the Bureau of Animal Industry, Washington, D.C.

Healthy 6-week-old New Hampshire Red chickens were used, and all birds were identified by wing bands. Serum from these chickens showed no neutralizing antibodies for Newcastle disease when tested by the chick embryo neutralization test prior to the start of the experiment. Three hundred fifty-two chickens were vaccinated by injecting 0.2 cc of a 10% suspension of virus-bearing hamster brain into the base of the wattle. Forty infected hamster brains of the eighty-

second subculture were used for preparation of the vaccine, with physiological saline as the diluent. One hundred thirty-four unvaccinated chickens were placed with the vaccinated birds as room contact controls. All of the chickens were checked carefully each day. Table I shows the observations during the 28-day period prior to challenge.

Three groups were then set up, each consisting of vaccinated birds, unvaccinated room control and normal susceptible birds of corresponding age. Group I was challenged with the virulent chick embryo propagated California strain, No. 11,914, subculture 18. Group II was challenged with the virulent chick embryo propagated Colorado strain, No. 8518, subculture 8. Group III was challenged with the virulent chick embryo propagated Connecticut strain, subculture 8. The results obtained after challenge are shown in

TABLE I.
Evaluation of Chicken Deaths Following Vaccination, Prior to Challenge.

	Vaccinated (352)	Room controls (134)
Coccidiosis	10	1
Newcastle	1	0
Smothered	1	2
Miscellaneous	15	2

¹ Reagan, Reginald, L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

² Reagan, Reginald, L., Lillie, Mary, G., Poelma, Leo J., and Brueckner, A. L., to be published.

TABLE II.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
 Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
 Route of challenge: Base of wattle. Date of challenge: 10/3/47
 Challenge dose: 0.5 cc of given dilutions.
 Challenge Virus (California Strain).

Group I	Dilutions								%	
	10-1		10-2		10-3		10-4			
	S*	D*	S	D	S	D	S	D	D	S
Vaccinated	18	2	15	5	17	3	17	3	16	84
Unvaccinated contact controls	2	8	7	3	4	6	3	7	60	40
Normal controls	3	7	5	5	6	4	3	7	57.5	42.5

* S—No noticeable symptoms.

D—Dead, also those showing Newcastle disease symptoms.

TABLE III.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
 Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
 Route of challenge: Base of wattle. Date of challenge: 10/3/47
 Challenge dose: 0.5 cc of given dilutions.
 Challenge Virus (Colorado Strain).

Group II	Dilutions								%	
	10-1		10-2		10-3		10-4			
	S*	D*	S	D	S	D	S	D	D	S
Vaccinated	31	0	19	1	17	3	19	0	4.44	95.56
Unvaccinated contact controls	5	6	1	9	6	4	3	7	63.4	36.6
Normal controls	7	3	7	3	6	4	8	2	30.0	70.0

* S—No noticeable symptoms.

D—Dead, also those showing typical Newcastle disease symptoms.

TABLE IV.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
 Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
 Route of challenge: Base of wattle. Date of challenge: 10/3/47
 Challenge dose: 0.5 cc of given dilutions.
 Challenge Virus (Connecticut Strain).

Group III	Dilutions								%	
	10-1		10-2		10-3		10-4			
	S*	D*	S	D	S	D	S	D	D	S
Vaccinated	13	1†	20	1	19	0	19	1	1.4	98.6
Unvaccinated contact controls	9	1	8	2	18	0	10	0	6.25	93.75
Normal controls	9	1	6	4	8	2	8	2	22.5	77.5

* S—No noticeable symptoms.

D—Dead, also those showing Newcastle disease symptoms.

† Crushed.

Tables II, III, and IV, respectively. The challenge was injected into the base of the wattle in various dilutions on the twenty-eighth day after vaccination. The results of these tests are also given in Tables II, III, and IV, respectively.

Table II shows that the living hamster-

adapted California strain provided good protection against the virulent egg-propagated California strain. The unvaccinated room controls and normal controls, challenged with the vaccinated chickens, showed approximately equal susceptibility.

In the case of the Colorado strain, No.

thrombin A."¹⁰ The presence of the factor in serum might explain Schofield's¹ and Rod-erick's² therapeutic findings—findings difficult to understand if deterioration of prothrombin alone were present in cases of dicumarol or toxic sweet clover poisoning.

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¹⁰ Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

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² Reagan, Reginald, L., Lillie, Mary, G., Poelma, Leo J., and Brueckner, A. L., to be published.

Phytopharmacological Experiments with Urethane.

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Ethyl carbamate or urethane has been known to pharmacologists for a long time. In fact, its physiological properties were first described by Schmiedeberg,¹ the father of modern pharmacology, who was the first to point out its narcotic properties.

At the beginning of the twentieth century it was quite extensively employed as an anesthetic for laboratory animals and as late as 1929 Macht and Hill² called attention to its usefulness for experimenting on small animals such as rats. Clinically, its usefulness was favorably reported towards the end of the nineteenth century as a hypnotic causing very little depression of the circulation and respiration.³⁻⁷ The drug became popular in medical practice until the introduction of the powerful barbiturates which supplanted urethane on account of its rather weak and uncertain hypnotic effects on human beings. Nevertheless, the drug has been occasionally employed in psychiatric practice.

More recently, a new property of this organic chemical compound has been discovered by Haddow and Sexton, who investigated the action of urethane in connection with their studies on the influence of various drugs on the growth of malignant tumors. These studies revealed that while ethyl carbamate produced inconclusive effects on the growth of malignant cells, it did produce a very

marked fall in the leucocyte count.

Prompted by this discovery, Paterson, Haddow, Thomas, and Watkinson⁸ investigated the effects of urethane on the blood of leukemia and noted in 32 cases of human leukemia a decrease in the total white blood cell count and the return to a normal differential pattern of the white cells. Similar findings were reported by Engstrom, Kirschbaum, and Mixer⁹ in their experiments with myelogenous leukemia in mice. More recently, similar studies were reported by Law concerning the effect of urethane on transplantable acute lymphoid leukemia, and further studies were described by Weir and Heinle.

Hirschboeck, Lindert, Chase, and Calvy¹⁰ found little effect produced by urethane on malignant tumors but they did observe marked action of the chemical on leukemic tissues leading to satisfactory remissions in some human patients.

The explanation of this interesting property exhibited by ethyl carbamate is still not clear. Law, studying the effects of urethane on transplantable lymphoid leukemia, concludes that immature lymphoblasts are significantly decreased in leukemia patients and hemoglobin values remain at higher levels in patients treated with the drug. He also found that the growth of the local subcutaneous tumor masses was retarded.¹¹ Kirschbaum and Lu demonstrated that urethane decreased the number of mitotic figures in the marrow

¹ Schmiedeberg, O., *Arch. f. exp. Path. u. Pharmacol.*, 1895, **20**, 203.

² Macht, D. I., and Hill, P. S., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1929, **26**, 871.

³ Andrews, J. B., *Am. J. Insan.*, 1886, **43**, 256.

⁴ Huebard, H., *Bull. gen. de therap.*, 1886, **110**, 103.

⁵ Hübner, C., and Sticker, G., *Deutsche med. Wchnschr.*, 1886, **12**, 236.

⁶ Sieveking, E. H., and Manifold, C. C., *Brit. M. J.*, 1886, **2**, 108.

⁷ Binet, P., *Rev. med. de la Suisse Rom.*, 1893, **13**, 540, 628.

⁸ Paterson, E., Haddow, A., Thomas, I., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

⁹ Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

¹⁰ Hirschboeck, John S., Lindert, M. C. F., Chase, Jules, and Calvy, Thomas L., *J. A. M. A.*, 1948, **136**, 90.

¹¹ Law, L. W., *Proc. Nat. Acad. Science*, 1947, **33**, 204.

TABLE V.
Unchallenged Contact Control Chickens.

Exposure strain	Vaccinated		Unvaccinated	
	Survivors c/o NC symptoms	Newcastle symptoms	Survivors c/o NC symptoms	Newcastle symptoms
California	30	0	15	5
Colorado	30	0	17	3
Connecticut	22	0	20	0

8518, the challenge virus was not as virulent as the California strain, as shown in Table III. The calculated amount of protection appears low, although only 4% of the vaccinated group failed to withstand the challenge. The unvaccinated room controls challenged with the Colorado strain showed greater susceptibility than normal controls challenged similarly.

The challenge virus of the Connecticut strain was of slightly lower virulence than that of the Colorado strain, as shown in Table IV. Since only one per cent of the vaccinated group showed typical Newcastle symptoms after challenge, the amount of protection is significant. The unvaccinated room control group showed much less susceptibility than the normal control birds in this group.

A contact exposure experiment was also conducted. Vaccinated and normal chickens of corresponding ages were placed with the control virus-challenged birds of the 10^{-1} and 10^{-2} dilutions of the California, Colorado, and Connecticut groups respectively, as shown in Table V. The results of this exposure are shown in the same table. None of the vaccinated birds succumbed. The contact exposure to Connecticut virus was not sufficiently great to cause disease in the normal unvaccinated birds. Comparatively few unvaccinated contact birds developed Newcastle disease in the California and Colorado groups. All unvaccinated chickens for this contact

exposure were brought to the laboratory the day of challenge, at which time they were placed with their respective groups.

Conclusion. Hamster-adapted Newcastle virus of the 82nd subculture immunizes young susceptible chickens against virulent egg-adapted California strain No. 11,914 and against 2 less virulent strains, the Colorado strain No. 8518 and the Connecticut strain, subculture 8.

Summary. The pathogenicity of the 82nd subculture of the hamster-adapted California strain of Newcastle virus used as an immunizing agent was negligible in this experiment.

Through injection of the living hamster-adapted California strain No. 11,914, of Newcastle virus, young chickens were successfully immunized against the egg-adapted California, Colorado, and Connecticut strains. The modified Newcastle virus also produced exceptionally good protection in chickens to contact with chickens infected with all above-mentioned strains.

Susceptible birds, used as contact controls with birds injected with modified hamster-adapted Newcastle virus, failed to show clinical evidence of spread of this modified strain. There appeared to be some resistance to challenge injection in those birds challenged with the Connecticut strain, as compared with the normal unvaccinated control chickens of this group.

TABLE I.
 Influence of Urethane on Growth of Seedlings.

Normal Plants.		Yarovized Plants.		Irradiated Plants, 500 r	
Urethane	Phytotoxic index, %	Urethane	Phytotoxic index, %	Urethane	Phytotoxic index, %
1:10,000	105	1:100,000	86	1:100,000	92
1: 5,000	100	1: 75,000	76	1: 50,000	87
1: 2,000	100	1: 60,000	70	1: 25,000	65
1: 1,000	98	1: 50,000	58	1: 10,000	60
1: 500	90	1: 10,000	49	1: 5,000	50
1: 250	72	1: 5,000	36	1: 1,000	25
1: 100	60	1: 1,000	24		
		1: 500	17		

ture thermostat for 24 hours at 60°F (15°C) and the influence of the drug on root growth was compared in the usual manner.

It was found that vernalized plants were extremely sensitive to the effects of urethane, whereas normal seedlings were very little affected by concentrations of 1:1000. The vernalized seedlings were sensitive to solutions of as little as 1:100,000. This is well illustrated in Table I.

A third method of studying urethane on what may be termed abnormal plant cells was by the employment of seedlings treated with deeply penetrating X-rays. Seeds of *Lupinus albus* were germinated in sphagnum and when 2 days old were radiated with X-rays filtered through a composite filter consisting of 1 mm of aluminum and 2 mm of copper. The apparatus used was a therapy machine operated on 200 Kv on a current of 20 ma, the plants being at a target distance of 50 cm. The dosage employed for the most part in the present experiments was 500 r. The results obtained are shown in the third column of Table I. It will be noted that such irradiated seedlings were also rendered much more sensitive to the effects of ethyl carbamate than normal seedlings.

Comment. There is considerable literature dealing with the inhibitory or suppressive action of urethane, phenyl carbamate, and other carbamates on the growth processes of bacteria, protozoa, and various animal tissues, as well as plant tissues. Templeman and Sexton¹⁸ studied the effects of such compounds upon cereals and other plant species. Lefèvre¹⁹

studied the effects of urethane on wheat seedlings, oats, barley, and rye. Both of the above investigators noted cytological alterations, blocking of mitosis, and other profound changes in the plant soil protoplasm produced by the drugs, not unlike those produced by colchicine. As far as the author is aware, however, no pharmacological studies were reported in which abnormal seedlings were compared with normal ones in respect to their reactions to urethane. The present findings, therefore, are of considerable interest, and are of particular interest in connection with the effects produced by urethane on malignant tumor cells and on leukemia blood cells of animals as compared with normal animal tissue cells. It is not at all uncommon in pharmacology to find a difference in reaction to drugs or poisons between normal animal cells and pathological animal tissues.²⁰ The same has been shown by the present author to be true of plant cells and is strikingly illustrated in the present investigation which reveals such a marked difference in response to urethane by the normal *Lupinus* seedlings and seedlings which have been yarovized on the one hand and irradiated on the other. These studies should encourage further investigations on the effects of various chemicals and drugs on abnormal cells both of animals and plants with a view of finding possibly some valuable agents that may inhibit the growth of malignant issues.

Summary. Solutions of ethyl carbamate or urethane were studied in different concentrations as regards their influence on root growth of *Lupinus albus* seedlings. It was found

¹⁸ Templeman, W. G., and Sexton, W. A., *Nature*, 1945, 156, 630.

¹⁹ Lefèvre, J., *C. R. Acad. Sci. Paris*, 1939, 208, 391.

²⁰ Macht, D. I., and Ting, G. C., *J. Pharm. and Exp. Therap.*, 1921, 18, 111.

myeloid cells and reduced the total number of circulating white cells, especially myeloblasts, in transplanted myeloid leukemia of F strain mice, while Moeschlin¹² claims that in the treatment of leukemia with urethane there is noticed an elective inhibition of the karyokinesis of leukemic neoplastic cells.

The present author for the past 25 years studied the action of various chemicals, drugs, and toxins on plant cells as compared with animal cells. These studies which have been carried on in a quantitative experimental fashion on various plants and especially on the root growth of *Lupinus albus* seedlings have been described in detail.^{13,14} It was deemed worthwhile, therefore, to examine the effects of urethane by phytopharmacological methods and the results are reported briefly. This was deemed especially worthwhile because of the recent announcement by Giles that radioactivity produces marked changes in plant cells.¹⁵

Method. The biological criterion employed was the elongation or growth of the well-defined straight roots of *Lupinus* seedlings, studied by the author's phytopharmacological methods, details of which have been published. The procedure was briefly as follows:

Seeds of *Lupinus albus*, large variety, were soaked in tap water overnight and planted in finely ground sphagnum moss containing adequate moisture. After germination, the growth of selected seedlings with roots 35 to 50 mm long was studied in a solution consisting of equal parts of distilled water and Shive physiological saline.¹⁶ At least 20 seedlings, placed in hard-glass test tubes, were employed for the control solution and for each drug solution, in the different concentrations used. The sharply defined straight roots were measured at the beginning and end of every experiment, and the ratio of growth of the seedling roots in the various drug solutions is ex-

pressed as a percentage of their growth in the control solution according to the formula,

$$\text{Index of growth} = \frac{\bar{X}}{\bar{N}} \times 100$$

in which \bar{N} represents average growth of controls and \bar{X} the average growth in the drug solution. All of the seedlings were allowed to grow for 24 hours in the dark at 15°C.

Results. In Table I are shown the results obtained with different concentrations of urethane on the growth of *Lupinus albus* seedlings studied in a manner described above. It will be seen that normal seedlings are very little affected by the drug. Even solutions 1:1000 are inhibited only slightly, the index of growth being on the average above 90%.

A very different effect of urethane was obtained by the use of seedlings raised in a different manner. The present writer has been making studies on the comparative reaction of normal seedlings on the one hand and so-called vernalized or "yarovized" seedlings of *Lupinus albus*. Yarovization is a term introduced by Russian plant-physiologists to describe a phenomenon produced by exposing plants to extreme variations of temperature.¹⁷ A simple and efficient method for obtaining yarovized seedlings is to expose them to quite low temperature but not such as would freeze the plant. Such plants obviously are markedly changed in their metabolism and the author has found that such seedlings may respond differently to certain blood sera and drugs from the responses given by normal seedlings, even though both the normal and the previously yarovized plants are allowed to grow at normal temperatures when the phytopharmacological experiment is performed. In the present research, *Lupinus* seedlings were germinated in sphagnum moss and when they were 2 days old, the jar with the young seedlings was placed in a refrigerator for 24 hours at 42°F (5.5°C). These seedlings were then employed simultaneously with other seedlings reared at a normal temperature of 70° and exposed to the action of various concentrations of urethane. Both sets of plants were placed in a low tempera-

¹² Moeschlin, Sven, *Experientia*, 1947, **3**, 195.

¹³ Macht, D. I., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 217.

¹⁴ Macht, D. I., and Macht, M. B., *J. Lab. and Clin. Med.*, 1941, **26**, 597.

¹⁵ Giles, Norman F., Jr., *Proc. Nat. Acad. Sci.*, 1947, **33**, 283.

¹⁶ Shive, J. W., *Physiol. Rev.*, 1915, **1**, 327.

¹⁷ Miller, E. C., *Plant Physiology*, 2nd Edition, 1938.

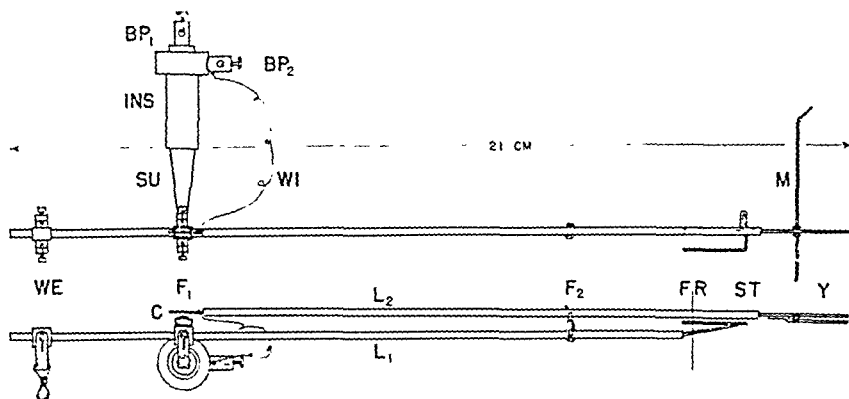


FIG. 1.
Contacting lever system. See text.

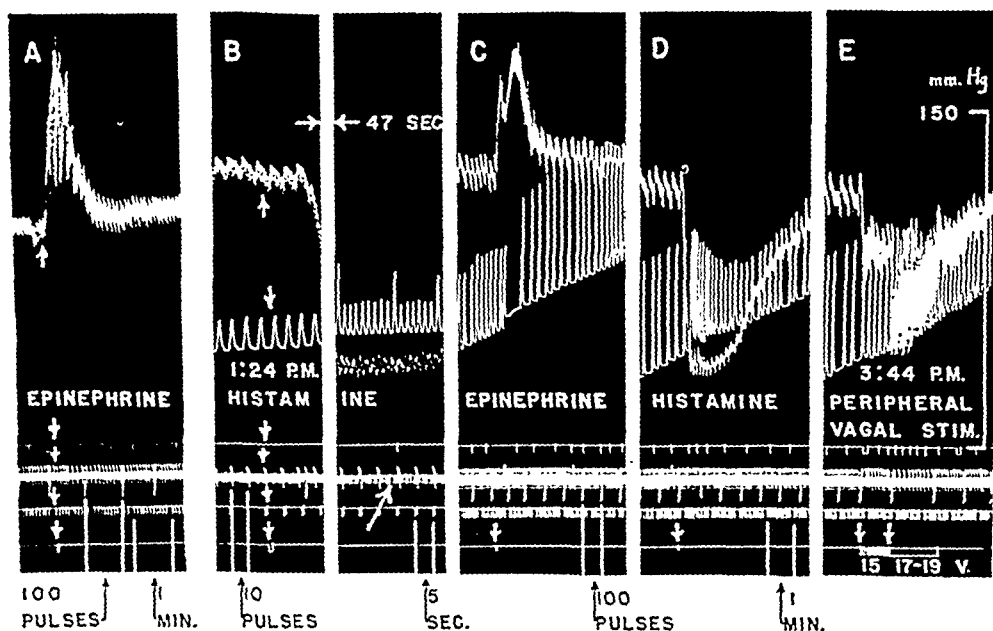


FIG. 2.

Illustrations of pulse-frequency recording under various conditions and recording speeds. *A* is from one phenobarbitalized dog, *B* to *E* from another. Small vertical arrows indicate simultaneous points. Vagus nerves were cut between *B* and *C*. Recordings from below upward are signal, time, pulse frequency, respiratory minute volume, spirometer, and femoral arterial manometer engaging the contacting lever. Large diagonal arrow indicates failure of one individual pulse to register (see text).

lever into three tines which are displaced, respectively, by (a) the ratchet, (b) a notch in the units-wheel, and (c) the lug on the tens-wheel of the counter mechanism. On a single record, individual pulses, tens, and hundreds are thus characterized by suitable amplitudes and/or directions of lever-movement. This summing recorder does not require calibration, and is more compact

and more easily devised than a Zeitschreiber.²⁻⁶ The latter, equally well adaptable to

² Fleisch, A., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Urban & Schwarzenberg, Berlin and Wien, 1935, Abt. V, Teil 8, 1935, p. 905.

³ Gesell, R., *Science*, 1934, **70**, 275.

⁴ Gaddum, J. H., and Kwiatkowski, H., *J. Physiol.*, 1938, **94**, 87.

that whereas normal seedlings are very little affected by the drug, other seedlings of an abnormal type were very sensitive to urethane. Specifically, it was found that yarovized or vernalized seedlings are extremely sensitive to even dilute solutions of the drug and the

same was found to be true of seedlings which were irradiated with hard X-rays in doses of 500 r.*

* By courtesy of Dr. Marcus Ostro, Radiologist in Chief, Sinai Hospital, Baltimore.

16265

An Easily Assembled Pulse-Frequency Recorder.

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Continuous, automatic, kymographic recording of pulse frequency conserves time, recording paper, and workers' patience. The device to be described is easily assembled with ordinary laboratory tools and supplies.

As shown in Fig. 1, a heart-lever fulcrum (F_1) is equipped with a tight-fitting, hollow laboratory straw (carrying lever, L_1) bearing an adjustable counterpoise (WE) on the short arm, and the fulcrum (F_2) of a second straw (contacting lever, L_2) on the long arm. The counterpoise is a weighted heart-lever fulcrum. The fulcrum of the contacting lever is a piece of aluminum (lever) wire, wound about and cemented (Duco) to the carrying lever, and passing as a bearing pin in a horizontal hole through the contacting lever. Into one end of the contacting lever is cemented a soldered-wire slip-yolk (Y) which engages the cross (recording) wire (M) of an ordinary recording mercury manometer. Into the other end is cemented a platinum wire which makes contact (C) with a drop of mercury in a tiny platinum-foil cup soldered onto the fulcrum block of the carrying lever. The long arm of the carrying lever is extended with an aluminum wire shaped (a) to serve as an adjustable stop (ST) for the contacting lever, and (b) as a lateral friction surface of the carrying lever against a lightly contacting, silk thread (FR) rendered slightly taut by the tension of a rubber band at one end. Contact C closes a circuit between the grounded and insulated (INS) binding posts (BP_1 and BP_2) on the insulation-sheathed

supporting rod (SU). The conducting wire (WI) from the platinum wire must be fine and flexible.

The contacting lever is balanced on its fulcrum by a suitable combination of fulcrum position and lengths of wires inserted into its ends. The entire system is then balanced on the carrying fulcrum by adjustment of counterpoise, WE. Engagement with the manometer thus has no significant influence on the latter's mean recording position.

To facilitate adjustments, universally adjustable clamps are used for mounting the device proper and the rods between which the friction thread is strung. The stop (ST) for the contacting lever is adjusted by bending so that the lever's freedom of excursion does not exceed the smallest systolic excursion of the manometer apt to be experienced (short of terminal cardio-vascular failure). The light tension of the friction thread is adjusted to just hold the carrying lever at the position to which it is brought by the excess of the manometer excursion beyond the contacting lever's short range. Automatic compensation thus occurs to changing mean and pulse pressures.

In present use, this contactor activates, through a standard-type electronic relay, a modification of the "counter-recorder" previously described.¹ The modification consists of branching the power end of the recording

¹ Winder, C. V., and Moore, V. A., *J. Lab. and Clin. Med.*, 1945, 30, 894.

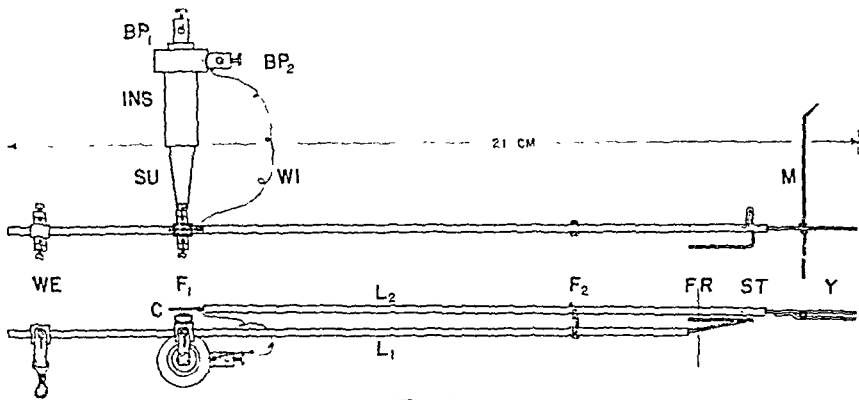


FIG. 1.
Contacting lever system. See text.

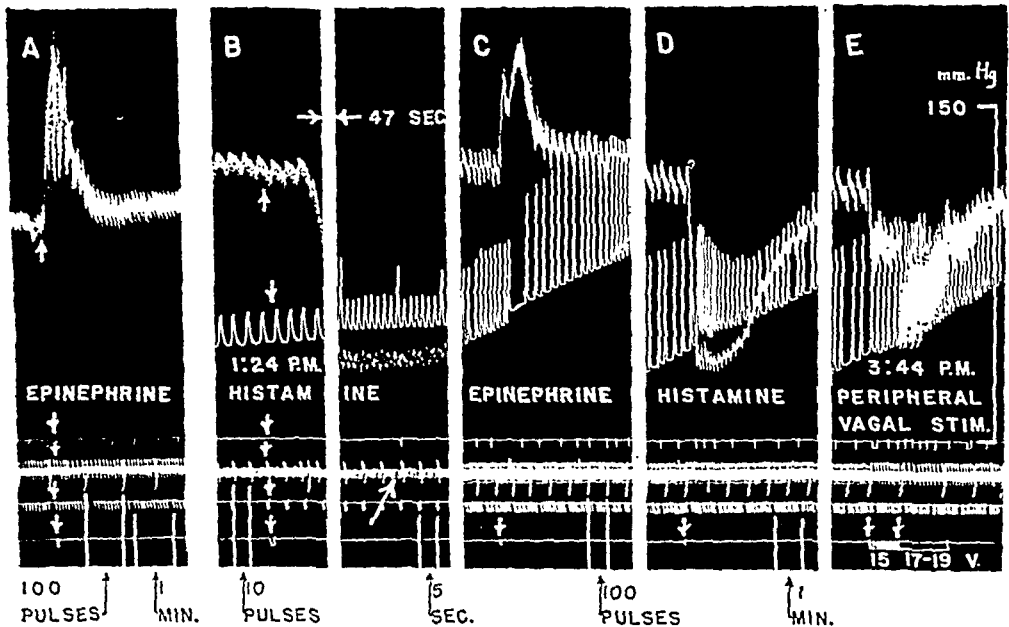


FIG. 2.

Illustrations of pulse-frequency recording under various conditions and recording speeds. *A* is from one phenobarbitalized dog, *B* to *E* from another. Small vertical arrows indicate simultaneous points. Vagus nerves were cut between *B* and *C*. Recordings from below upward are signal, time, pulse frequency, respiratory minute volume, spirometer, and femoral arterial manometer engaging the contacting lever. Large diagonal arrow indicates failure of one individual pulse to register (see text).

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² Fleisch, A., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Urban & Schwarzenberg, Berlin and Wien, 1935, Abt. V, Teil 8, 1935, p. 905.

³ Gesell, R., *Science*, 1934, 79, 275.

⁴ Gaddum, J. H., and Kwiatkowski, H., *J. Physiol.*, 1938, 94, 87.

the present contactor, does not require exact reference to a simultaneous record of time, indicates instantaneous changes in frequency, and provides a ready-made graph of the (reciprocal) frequency course. Junkmann's⁷ original Impulszähler falls into the latter category except for *direct* frequency plotting and for lack of instantaneous information.

As illustrated in Fig. 2, with optimal adjustment, the systolic excursions of the manometer are selectively registered during widely and abruptly varying patterns of respiratory pressure waves, pulse pressure, and mean pressure. The limits of fidelity are those of the mercury manometer, as illustrated by the individual unregistered systole in Fig. 2B (arrow), where a very low pulse oscillation was masked by relatively vigorous respiratory waves during histaminic hypotensive dyspnea.

⁵ Druckrey, H., and Uhlig, P., *Arch. f. exp. Path. u. Pharmacol.*, 1941, **198**, 74.

⁶ Genuit, H., and Ruppert, H., *Arch. f. exp. Path. u. Pharmacol.*, 1943, **200**, 684.

⁷ Junkmann, K., *Arch. f. exp. Path. u. Pharmacol.*, 1927, **120**, 314.

The principle of automatic compensation to varying hemodynamics, fully present in the contacting instrument of Genuit and Ruppert⁶ which inspired the present simplification, was lacking in Fleisch's Pulspelotte and partially present in such manner as to operate against sensitivity in Nyboer's⁸ contactor. Electronic cardi tachometers employing the cardio-electric R-wave are, of course, inherently independent of hemodynamics.

It will be apparent that the contactor described is adaptable to many oscillatory phenomena, with provision of a simple engagement for the contacting lever.

Summary. A simple contacting lever, mounted on a carrying lever and engaged with a mercury-manometer cross-wire, selectively registers systolic pulsations. It is used to activate, through a relay, a simple counter-recorder,¹ improved. Individual pulses, tens, and hundreds (etc.) are characterized by amplitude and/or direction of excursion of a single recording lever. Operation and limitations are illustrated.

⁸ Nyboer, J., *Am. J. Physiol.*, 1933, **106**, 204.

16266

Alkaline Phosphatase Activity and Basophilia in Hepatic Cells Following Administration of Butter Yellow to Rats.*

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A cytochemical study¹ has been reported previously of the activity of phosphatase (at pH 5.0) in the cytoplasm of motor neurons during their regeneration. A well-defined increase was found to be associated with the

dissolution of cellular basophilic material, chromatolysis, and its regeneration, chromatogenesis.

The alterations in phosphatase activity previously described were in cells which do not divide. It was desirable, therefore, to extend the observations to include regenerating cells in which mitosis can be investigated.

* Presented at the Fourth International Cancer Research Congress, St. Louis, September 6, 1947. A rather similar study was reported by N. Bucher and A. Glinos at this Congress.

[†] This work was supported by a Fellowship Grant of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

¹ Bodian, D., and Mellors, R. C., *J. Exp. Med.*, 1945, **81**, 469.

There are presented herewith the results of a study of alkaline phosphatase activity as correlated with basophilia in the hepatic cells of rats fed butter yellow (*p*-dimethylaminoazobenzene) in a diet which renders the liver of the animal susceptible to consequent cancer production.

A large part of the cytoplasmic basophilic substance is contained in prominent structural entities, called Nissl bodies in the case of the nerve cell. These structures have been shown to consist of a protein matrix with a basophilic component which can be removed by ribonuclease activity.^{2,3} They absorb ultraviolet light at 2600 Å,^{3,4} and do not react by Feulgen's method. It can be concluded, therefore, that they contain nucleic acid of the ribose type.

Opie⁵ described the mobilization of basophilic material in the cytoplasm of liver cells in which degenerative, regenerative, and proliferative changes had been induced by butter yellow. This material was shown by Opie and Lavin⁶ to have the characteristics of ribonucleic acid.

The demonstration of alkaline phosphatase in large granules separated from the cytoplasm by Claude⁷ and in residual chromosomes isolated from the nucleus by Mirsky and Ris⁸ indicates an association of this enzyme, or group of enzymes, with structural and functional components of the cell.

Methods. Thirty-three albino rats (Sherman strain) were offered a diet of unpolished rice supplemented with fresh carrots. Butter yellow, *p*-dimethylaminoazobenzene,⁹ was included at a level of 0.06%. Six normal rats of comparable age and weight, offered the same diet but without butter yellow, were studied as controls. At approximately weekly intervals from 7 to 258 days, the animals were killed and studied histologically. The weights at death were from 85 to 380 g.

Thin slices of liver were fixed immediately

in ice-cold acetone, imbedded in paraffin, and cut in serial sections. The method of Gomori¹⁰ was employed for the histological demonstration of alkaline phosphatase activity at pH 9.0, temperature 37°C, and period of incubation from 1 to 3 hours. A variety of substrates were used: glycerophosphate, fructose diphosphate, adenylic acid, and ribonucleic acid (from yeast). Adjacent sections were stained by a variety of technics, the most useful of which was that of Giemsa (Wohlbach's modification).¹¹ In addition, sections were studied for basophilia, when stained with methylene blue and toluidine blue under controlled conditions,¹² before and after incubation in a solution of purified ribonuclease,¹³ (enzyme concentration 0.1 mg/cc, pH 6.75, temperature 58°C, duration 2 hours) and desoxyribonuclease,¹⁴ (enzyme concentration 0.1 mg/cc, pH 6.75, Mg ion 0.003 M., temperature 37°C, duration 2 hours).

Results. Although acetone fixation is not ideal, basophilic bodies were prominent in the cytoplasm of the normal rat liver cells stained by Giemsa's procedure. (Fig. 1A). Alkaline phosphatase activity was uniformly slight in both the cytoplasm and the nucleus of these cells (Fig. 1B).

After the administration of butter yellow, however, degeneration with chromatolysis and regeneration with chromatogenesis were present in the cytoplasm essentially as described by Opie.⁵

Early focal chromatolytic changes were demonstrable after 24 days, and were prominent after 45 days, when some cells had lost much or all of their cytoplasmic basophilia and, in some instances, the staining properties of their nuclei as well. After 51 days, basophilia as evidence of chromatogenesis was seen in some cells, although advanced chromatolysis was still predominant. At 95

² Brachet, J., *C. R. Soc. Biol.*, 1940, **133**, 88.

³ Gersh, I., and Bodian, D., *J. Cell. and Comp. Physiol.*, 1943, **21**, 253.

⁴ Landstrom, H., Caspersson, T., and Wohlfart, G., *Z. mikr-anat. Forsch.*, 1941, **40**, 534.

⁵ Opie, E. L., *J. Exp. Med.*, 1946, **84**, 91.

⁶ Opie, E. L., and Lavin, G. I., *J. Exp. Med.*, 1946, **84**, 107.

⁷ Claude, A., *A. A. A. S. Research Conference on Cancer* (1944), Washington, D.C., A. A. A. S., 1945, 223.

⁸ Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31**, 7.

⁹ Sugimura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

¹⁰ Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 23.

¹¹ Mallory, F. B., *Pathological Technique*, Philadelphia, W. B. Saunders Co., 1938, 195.

¹² Dempsey, E. W., and Singer, M., *Endocrinol.*, 1946, **38**, 270.

¹³ Kindly supplied by Dr. M. Kunitz.

¹⁴ Kunitz, M., *J. Gen. Physiol.*, 1940, **20**, 15.

¹⁵ McCarthy, M., *J. Gen. Physiol.*, 1946, **29**, 123.

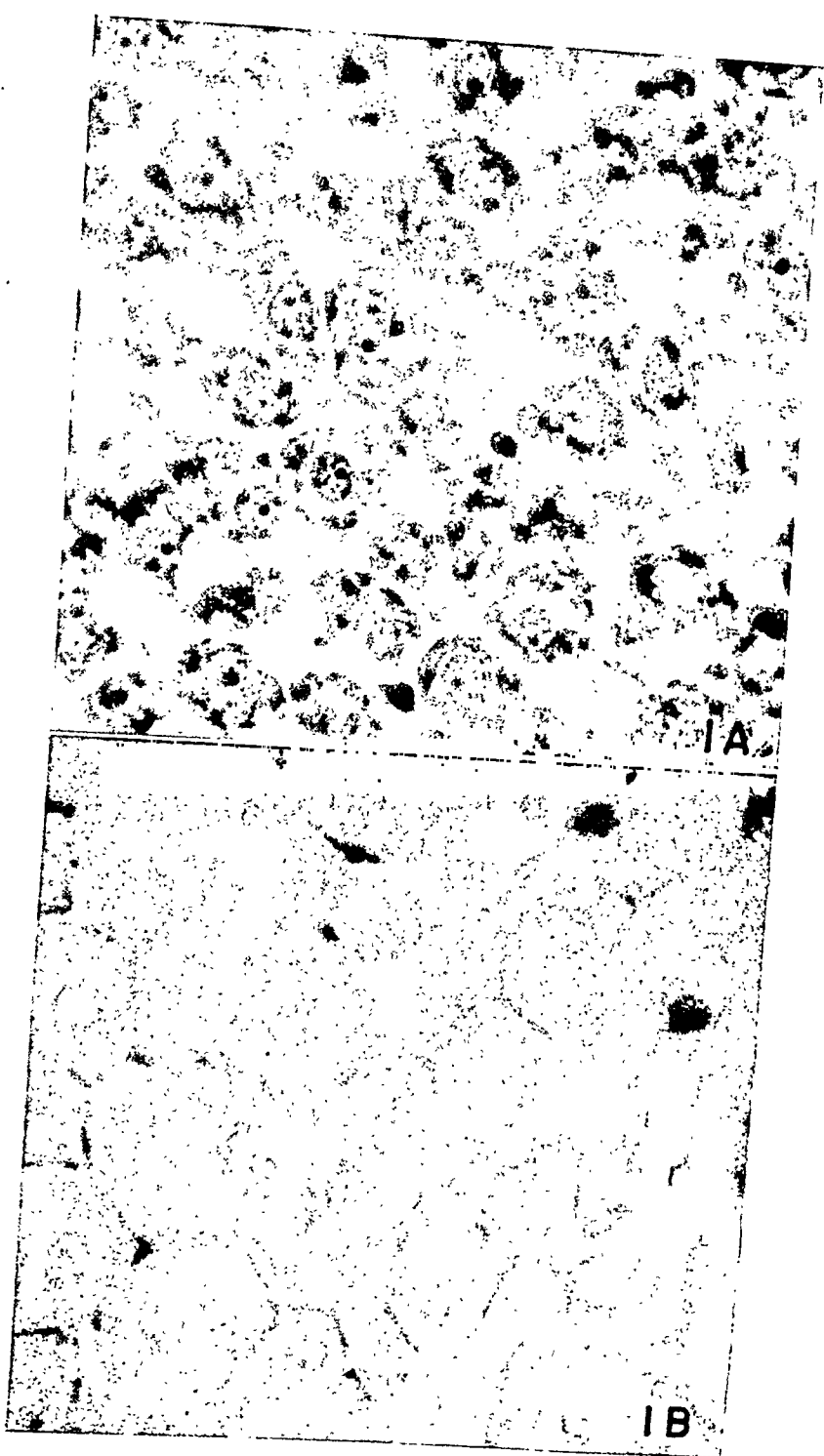


FIG 1A. Normal hepatic cells containing cytoplasmic basophilic material. Acetone fixation. Paraffin section 4 microns. Giemsa (Wolbach's). 500 X.

FIG. 1B. Normal hepatic cells with slight alkaline phosphatase activity. More conspicuous activity in biliary capillaries. Acetone fixation. Paraffin section 5 microns. Gomori's method. Glycerophosphate substrate, pH 9.0, 3 hours' incubation at 37°C. 500 X.

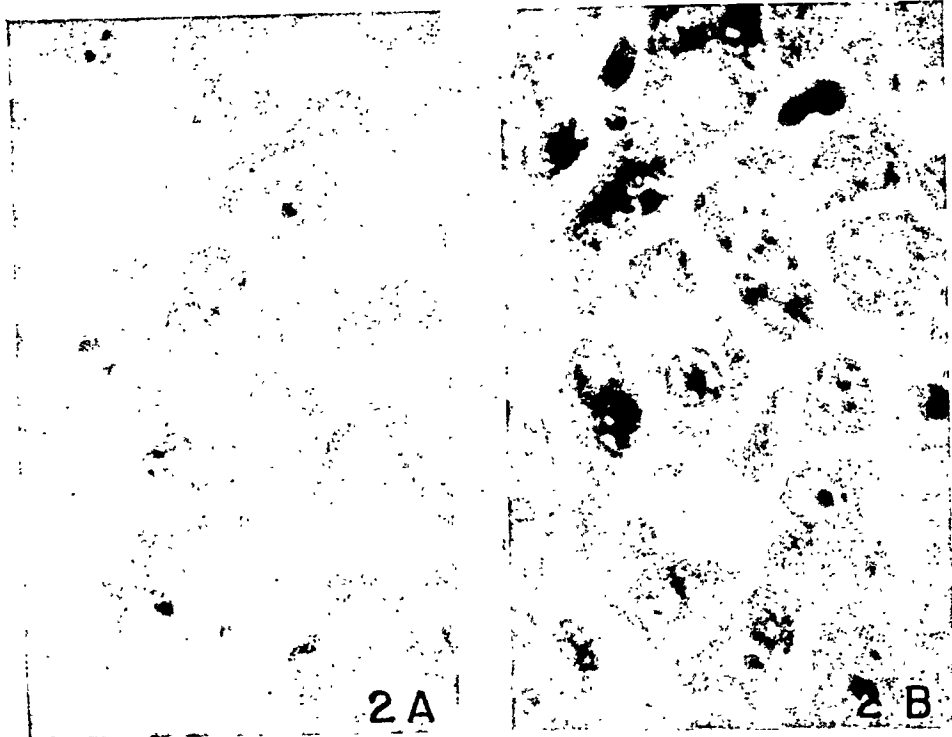


FIG. 2A. Degeneration and chromatolysis in hepatic cells after 95 days of butter yellow administration. Acetone fixation. Paraffin section 4 microns. Giemsa (Wolbach's). 500 X.

FIG. 2B. Same section as 2A. Focus of regeneration and chromatogenesis in hepatic cells, with prominent cytoplasmic basophilia.

days, cytoplasmic basophilia was intense in foci of regeneration and hyperplasia (Fig. 2B) in sharp contrast to the faint basophilia of cells with persistent evidence of degeneration and necrosis (Fig. 2A). After 197 days, focal basophilic regeneration and hyperplasia were associated with hepatoma, cholangioma, and adenocarcinoma.

A slight tendency toward decreased alkaline phosphatase activity at pH 9.0 was present in association with the hepatic chromatolysis (Fig. 3A). In contrast, an increased activity was frequently present, both in the nucleus (nuclear membrane, chromatin, nucleolus, dividing chromosomes) and to a lesser degree in the cytoplasm in foci of basophilic hyperplastic and proliferating cells (Fig. 3B). This conclusion was established by direct comparisons of sections stained for alkaline phosphatase activity with adjacent sections stained by the Giemsa procedure. In these comparisons, it was clear that, although a good

correlation could be drawn between basophilic hyperplasia of parenchymatous cells and a distinct increase in phosphatase activity, the findings were not absolutely uniform.

Observations of cells other than parenchymatous under the conditions of these experiments were of interest. In the epithelium of the bile ducts, no significant alteration in phosphatase activity occurred either during proliferation or actual hyperplasia. Intense, although not necessarily increased, phosphatase activity distinguished mast cells, sometimes present in abundance in the connective tissue around proliferating bile ducts. Conspicuous activity was occasionally present in the sinusoidal endothelium and usually present in the endothelium of the small blood vessels. Moderate activity was in evidence in proliferating fibroblasts in the stroma of cirrhosis and cholangiofibrosis.

In hepatoma, cholangioma, and metastasizing adenocarcinoma, the phosphatase activity

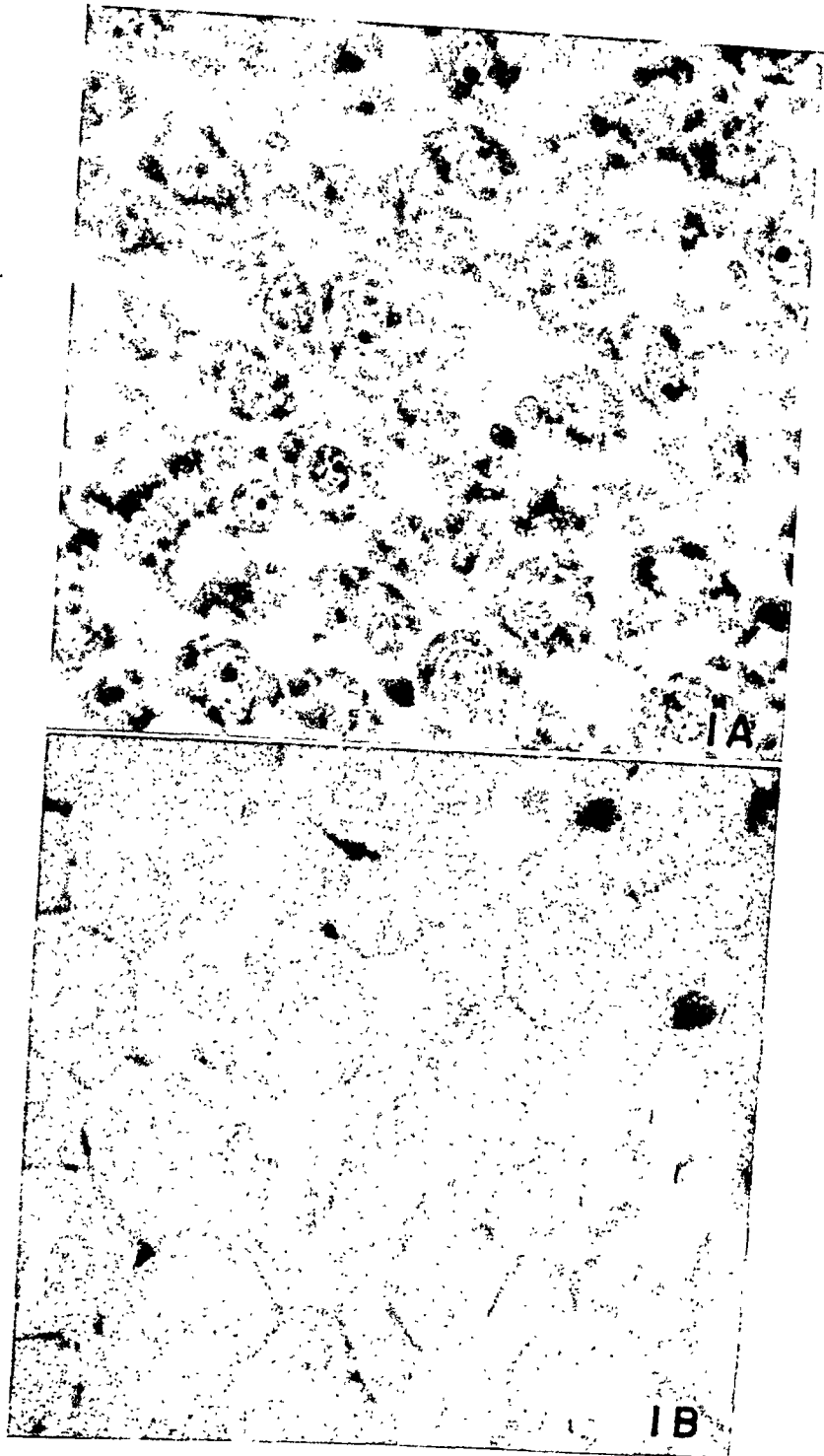


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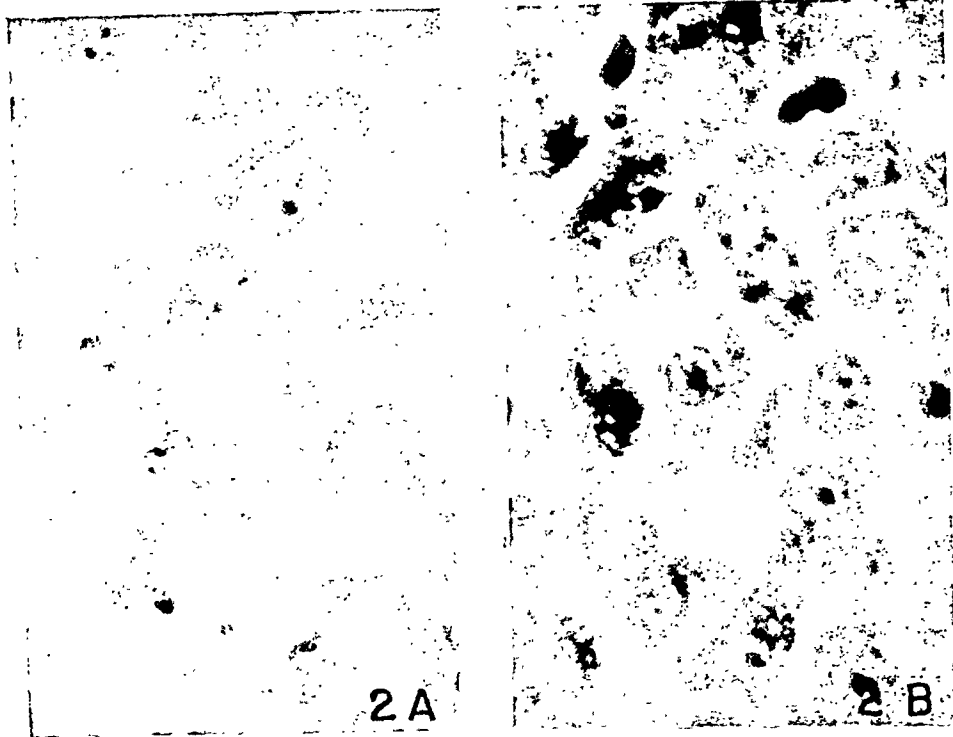


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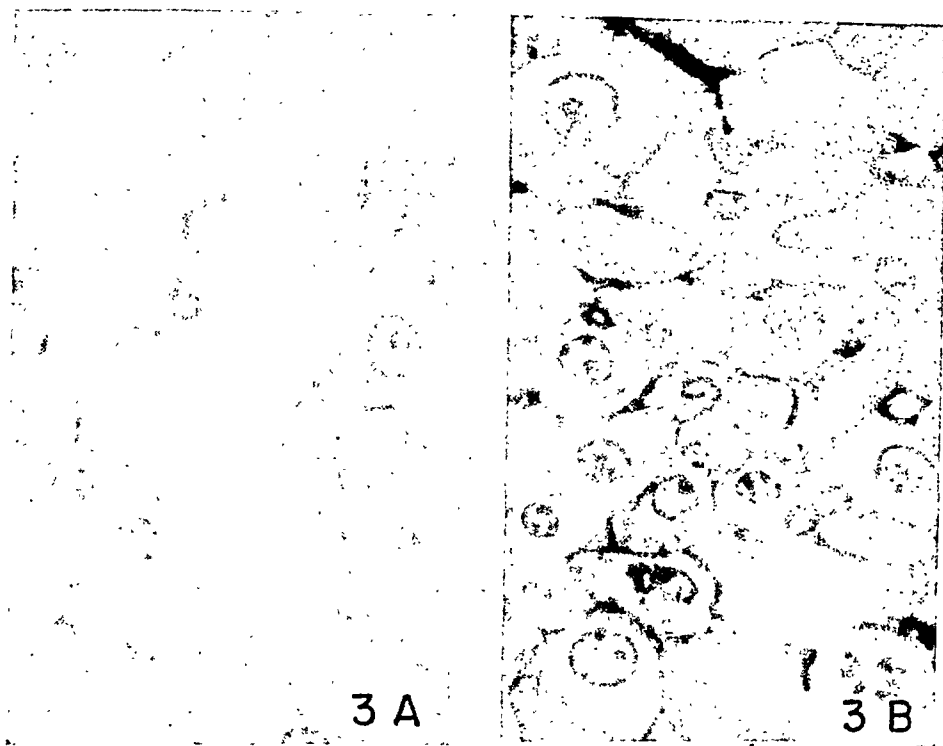


FIG. 3A. Section adjacent to 2A. Same area of degenerating and chromatolytic hepatic cells as shown in 2A, with very little alkaline phosphatase activity. Acetone fixation. Paraffin section 4 microns. Gomori's method. Glycerophosphate substrate, pH 9.0, 3 hours' incubation at 37°C. 500 X.

FIG. 3B. Same section as 3A. Same focus of regeneration and chromatogenesis as shown in 2B, with increased alkaline phosphatase activity in nucleus (membrane and chromatin), chromosomes, and, to a lesser extent, in cytoplasm of hepatic cells. Activity is conspicuous also in a bile capillary.

ity, variable and focal in distribution, occurred in the tumor cells, particularly in those which appeared to be degenerated or to be associated with necrotic debris.¹⁵

Summary and Conclusions. As with the reformation of Nissl substance in the motor neuron, the resynthesis of nucleic acid in the

cytoplasm of the regenerating hepatic cell, injured by butter yellow, is accompanied, during certain phases of the reaction at least, by an enhanced activity of alkaline phosphatase. The cellular distribution of the alteration is principally cytoplasmic in the nerve cell, with the optimum pH in the acid range, and nuclear in the hepatic cell, with the optimum pH in the alkaline range.

¹⁵ Kabat, E. A., and Farth, J., *Am. J. Path.*, 1941. 17, 303.

Diagnosis of Pregnancy by Cytologic Criteria in Catheterized Urine.*

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Pregnancy causes marked proliferation and growth in the epithelium of the vagina and the cervix.¹ This results in characteristic cytologic changes, which permit the diagnosis of pregnancy through the microscopic examination of fluid aspirated from the vagina. The use of the vaginal smear in diagnosing pregnancy was advocated by me in 1925.²

The extreme growth of the epithelium of the vagina and the cervix during gestation is accompanied by a considerable increase in the secretion of glycogen. Glycogenic cells become thus very prominent in the vaginal smear of pregnancy. The most typical of these cells are the so-called "navicular" cells,³ which have a definite diagnostic value. Their differentiation from corresponding cells of the normal cycle is possible through certain criteria such as enlargement and elongation of their nuclei and thickening of their surface membranes.

The vaginal smear method of diagnosing pregnancy, though morphologically sound, did not prove to be of great practical value as a routine diagnostic procedure. The identification of the "navicular cells of pregnancy" requires great experience and is not always dependable. It is often difficult to differentiate these cells from other forms of navicular cells found during the normal menstrual cycle or in certain types of secondary amenorrhea. Furthermore, other factors, such as intercourse or bacterial and parasitic infections may distort the vaginal smear picture, thus impairing its diagnostic value.

In the course of studies of smears prepared from urine sediment for the purpose of detecting cancer cells,^{4,5,6} I have been impressed by the greater cytologic uniformity of such smears in comparison with vaginal smears. The normal exfoliation in the urinary tract is much scantier than in the female genital tract and the variety in the types of desquamated cells is much more limited. The appearance of any significant cytologic changes can thus be noted much more readily in the "urine sediment" than in the vaginal smear.

The effect of the estrogenic hormone upon the cellular make-up of the urine sediment smear may be cited as an example. This effect is noticeable in the urine of women as well as of men. I first observed it in the case of a man receiving estrogenic therapy for a carcinoma of the prostate.⁵ It consists in the appearance of squamous acidophilic cells with a pyknotic nucleus and cytoplasmic granules greatly resembling the cells found in the vaginal smear either normally during the follicular phase or after estrogenic therapy. The recognition and identification of these cells is indeed easier and less equivocal in the "urine" than in the "vaginal" smear. This is because the vaginal smear contains almost invariably other non-specific squamous acidophilic small-nucleated cells which complicate the cytologic picture. The urine sediment smear may thus prove to be more accurate in assaying estrogens than the vaginal smear. The urine of women should be obtained by catheterization in order to prevent the admixture of vaginal cells.

More recently, my attention was directed

* Aided by a grant from the Commonwealth Fund.

¹ Stieve, H., *Z. f. mikrosk.-anat. Forsch.*, 1925, **3**, 3.

² Papanicolaou, G. N., *Proc. Soc. Exp. Biol. and Med.*, 1925, **22**, 436.

³ Papanicolaou, G. N., *Am. J. Anat.*, 1933, **52**, 3 (Supplement), 519.

⁴ Papanicolaou, G. N., and Marshall, V. F., *Science*, 1945, **101**, 2629, 519.

⁵ Papanicolaou, G. N., *J. Am. Med. Assn.*, 1946, **131**, 372.

⁶ Papanicolaou, G. N., *J. Urol.*, 1947, **57**, 375.



FIG. 1.

Photograph of drawings of cells of the navicular type found in smears prepared from the urine of pregnant women. $\times 400$.

toward the cytologic changes occurring in the urine of pregnant women. For this study catheterized urine was used, since voided urine is always contaminated with cervical, vaginal, and vulval cells. The pure catheterized urine contains normally a relatively small number of exfoliated cells, most of which are of the transitional type. The variety of cell types is limited. Leucocytes are, as a rule, scanty, except in cases of bacterial or parasitic infections of the urinary tract, which are much less frequent than those of the vagina and the cervix. Histiocytes are also relatively rare.

In the pregnancy urine, there is an apparent increase in the total number of desquamated cells. The cells appear singly or in clusters. Some of these acquire a characteristic form closely resembling that of the navicular cells found in the vaginal smear of pregnancy (Fig. 1). Here again, under the effect of the hormonal factors of pregnancy, the cytology of the urine sediment smear acquires a great similarity with that of the vaginal smear. Many of the cells show pronounced vacuolation and a displacement of the nucleus toward the periphery or one of the poles of the cell. The presence of glycogen within

the vacuoles may be demonstrated by special staining. There is, thus, evidence of an increased production of glycogen during gestation in the epithelium of the bladder, parallel to that of the epithelium of the cervix and the vagina. Leucocytes are, as a rule, inconspicuous. In general, the urine sediment smear shows a simpler and more uniform cytology than the vaginal smear and is much less contaminated with bacteria, leucocytes, and histiocytes.

Thus far, approximately 350 specimens of catheterized urine of women have been examined. Of these, 38 were from pregnant women. Cytologic changes characteristic of pregnancy were found in all specimens of this latter group. The earliest specimen examined was of a gestation of 14 weeks and the latest of seven months. A study of earlier as well as of more advanced cases is now in progress. Also, of abnormal conditions resulting from pregnancy, such as abortions, ectopics, hydatidiform moles, chorio-epitheliomas, etc.

The technic used in these studies is similar to the one used in cancer diagnosis. Catheterized urine, varying in quantity from 25 to 100 cc is mixed immediately with equal parts of 95% ethyl or with isopropyl alcohol and

centrifuged without much delay for 30 minutes at medium speed. The sediment is spread evenly on a number of slides which have been thinly coated with Mayer's albumen. The smears thus prepared are allowed to stand only for a short time until they begin to show dryness around the edge and then they are immersed in a solution of equal parts of 95% alcohol and ether.

After a fixation of at least 15 minutes the slides are carried through 80%, 70%, and 50% alcohols to distilled water.

The staining is as follows:

Harris Hematoxylin (which has been prepared without acetic acid, and diluted with an equal amount of distilled water) for 6 minutes.

Rinse in distilled water.

† EA65 is the same as EA36 with the difference that the Light Green is one-half strength (0.25% instead of 0.5%). It has the advantage of giving a lighter and more transparent staining which is very desirable in cancer diagnosis. The differentiation between acidophilic and basophilic cells is better with the EA36 (or EA50). This differentiation is more important in vaginal, endocervical, and endometrial smears. Therefore, EA36 (or EA50) is preferable for vaginal, endocervical, and endometrial smears, whereas EA65 is better adapted to other types of smears. Either one of these two stains can be used for all types of smears.

Dip in 0.5% HCl (aqueous solution) 6 times.

Place in running tap water for 6 minutes.

Rinse in distilled water, 50%, 70%, 80%, and 95% alcohols.

Stain in OG6 for 1½ minutes.

Rinse in 95% alcohol, 2 changes.

Stain in EA65† (procedure No. 267) for 1½ minutes or in EA36† (procedure No. 268).

Rinse in 95% alcohol (3 changes); absolute alcohol and xylol, equal amounts; xylol; mount with a coverslip.

Be sure that smears are never allowed to dry at any time.

Summary. Pregnancy causes distinct morphologic changes in the epithelium of the urinary tract which are reflected in the cytology of the "urine sediment smear." There is an appearance of certain characteristic cell forms such as cells of the "navicular" type corresponding to those described in the vaginal smear of pregnancy. The urine sediment smear provides, thus, a simple, rapid and dependable method for diagnosing pregnancy. It appears to be superior to the vaginal smear in that its cytology shows greater uniformity and is more distinctive. Its practical value as a routine diagnostic procedure and its applicability in the early diagnosis of pregnancy are still to be determined.

† Papanicolaou, G. N., *Science*, 1942, 95, 438.

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Streptomycin as an Essential Nutrilite.

GEOFFREY RAKE.

From the Squibb Institute for Medical Research, New Brunswick, N.J.

In consideration of the mode of action of streptomycin one of the possibilities explored has been that of its functioning as a metabolite antagonist. Little evidence of this has been forthcoming, however, and it was therefore with interest that one read the report of Miller and Bohnhoff¹ on their so-called B variant meningococcus. This variant (mutant) resembled the A variant (mutant)

in that both were resistant to, or developed in high concentrations of, streptomycin. The B mutant differed from the A in that it required streptomycin for multiplication either *in vitro*, or *in vivo* in the mouse, while the A mutant did not. The B mutant never

¹ Miller, C. P., and Bohnhoff, M., *Science*, 1947, 105, 620.

emerged in the presence of streptomycin concentrations less than 40 γ /ml, but once secured, its growth was promoted by 5 γ /ml but not less. Rare back-mutation to non-streptomycin-requiring forms occurred. A recent paper by Kushnick, Randles, Gray and Birkeland deals with variants (mutants) of *E. coli*, *P. aeruginosa* and *B. subtilis* requiring streptomycin.² All mutants showed this growth stimulation when glucose was present in the medium. In the case of *P. aeruginosa* sodium formate could replace the glucose. The case of *B. subtilis* was unusual in that the streptomycin-requiring mutants grew only under anaerobic conditions. Many papers in the literature^{3,4} demonstrate that the antibacterial action of streptomycin is greatly reduced by anaerobic conditions.

It has been possible to confirm the findings of Miller and Bohnhoff as regards streptomycin-requiring mutants of meningococci. These authors obtained such variants from 16 of 18 strains tested. They do not mention type distribution. In our case the mutants developed with a Type II (No. 763) and a Type II- α strain (No. 1054) but not, in limited tests, with a Type I strain (No. 1168).^{*} The medium we have used is a beef heart, glucose agar containing 4% fresh citrated rabbit blood⁵ on which the meningococcus grows readily. On such medium 2 colony forms have appeared similar to those described by Miller and Bohnhoff, but such difference in colony morphology has not been associated in our hands with as sharp differentiation as they found into colonies resistant to streptomycin but not requiring it as contrasted to colonies both resistant to and requiring the antibiotic. Furthermore, our mutant strains showed much less stability than that indicated by Miller and Bohnhoff

TABLE I. Serial Transfers of Mutant Strains of *E. coli*.

Transfer No.	Strains D56			Strain D57		
	Units/ml* streptomycin	Back-mutant†	Colony type	Units/ml* streptomycin	Back-mutant†	Colony type
1	5	Frequent	Undifferentiated	20	Frequent	Undifferentiated
2	20	"	"	80	"	"
3	80	"	Small	640	"	"
4	80	None	"	2560	None	"
5	320	Frequent	Undifferentiated	5120	Very few	Small
6	80	Very few	Small	1280	Frequent	Undifferentiated
7	40	Frequent	"	320	"	"
8	40	Very few	"	160	"	"
9	80	"	"	40	"	"
10	20	Frequent	Undifferentiated	40	None	Small
11	160	None	Small	20	"	"
12	160	"	Undifferentiated	20	"	"
13	40	"	"	20	"	"
14	10	"	"	10	"	Large
15	10	"	"	40	Very few	Undifferentiated
				10	"	"

* Concentration of streptomycin in units per ml on which colony grew and from which transfer was made.

† Frequency of colonies on control streptomycin-free medium; i.e., reversion to parent type no longer requiring streptomycin as essential metabolite.

² Kushnick, T., Randles, C. I., Gray, C. T., and Birkeland, J. M., *Science*, 1947, **106**, 587.

³ Donovick, R., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 224.

⁴ Bondi, A., Deitz, C. C., and Spaulding, E. H., *Science*, 1946, **103**, 399.

* My thanks are due to Dr. Sara E. Branham of the National Institute of Health, Bethesda, Md., for these strains.

⁵ Rake, G., *J. Exp. Med.*, 1934, **59**, 553.

TABLE II.
Optimal Concentrations of Streptomycin for *E. coli* Mutants.

Strain 56, 6th passage		Strain 57, 13th passage	
Streptomycin pool III Concentration/ml	Growth on plate	Streptomycin Concentration/ml	Growth on plate
20 u	+	5 u	0
40 u	+++	10 u	3 colonies
80 u	+++	20 u	+
160 u	++	40 u	4 colonies
320 u	0	80 u	0

and apparent back-mutation was too frequent to allow these strains to be used readily as a tool for further study. It may be that such unsatisfactory behavior is due, in part at least, to the fact that colonies for transfer were, after the first few transfers, selected from plates containing high concentrations of streptomycin (800 units/ml or greater).

At the same time that the work with meningococci was started we began investigation of 4 strains of *E. coli* of our own isolation. In limited tests one strain (H36) did not produce streptomycin-requiring mutants on beef heart agar⁶ (without rabbit blood), or on a fluid medium of the same beef heart infusion without the agar. The other 3 did. In the case of one strain, D11, there was still a marked tendency to back-mutation although it was less than in the case of the meningococcus mutants. This strain was maintained mostly by transfer from colonies growing at the higher concentrations of streptomycin (3,200 units/ml or greater). Both large and small colony types occurred but again there was no correlation of any such morphological appearance with a demand or no for streptomycin as an essential metabolite.

In the case of the two other strains of *E. coli*, D56 and D57, the later transfers in particular were made from colonies growing on agar with lower concentrations of streptomycin. As can be seen in Table I back-mutation became less frequent and the correlation of small colony with necessity for streptomycin was fairly good. The strains tended towards stability.

It is thus apparent that selection of the streptomycin-requiring mutants in pure form occurs more readily by colony picking from plates of lower, rather than of higher, concen-

trations of streptomycin. The exact reason for this is not clear but probably these mutants, though relatively resistant to streptomycin, are not as resistant as the mutants which, while growing in concentrations of streptomycin as high as 100,000 units/ml⁶ and possibly metabolizing the antibiotic, do not find it essential for their multiplication. Single cell isolation would be necessary for certainty on this point; its probability is borne out by the fact that the streptomycin-requiring mutants, when plated out on increasing concentrations of commercial streptomycin, or streptomycin A, showed an optimum which for the *E. coli* strains was around 20 to 80 units/ml (Table II). However, resistant forms could readily be selected which grew in much higher concentrations of streptomycin (5,000 units/ml or greater). These, which grow freely in absence of streptomycin, show limitation of growth at higher, but not at lower, concentrations of the antibiotic.

All the above work has been carried out on complex media. It was decided to attempt to adapt the mutants to a synthetic medium. After trial of several, that described by Cohen⁷ was finally adopted. For our purposes the pH was adjusted to 7.3 with NaOH before sterilization and a final pH of 7.0 was thus achieved. The medium was used mostly as a broth but also as an agar. Streptomycin-requiring mutants were obtained from both D56 and D57 strains of *E. coli* on such media. For most of the subsequent work D57 mutant (D57_M), carried in the fluid medium, has been used. As long as the broth cultures are not held under conditions allowing multipli-

⁶ Lapedes, D., Donovick, R., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 269.

⁷ Cohen, S. S., *J. Biol. Chem.*, 1947, **168**, 511.

emerged in the presence of streptomycin concentrations less than 40 γ /ml, but once secured, its growth was promoted by 5 γ /ml but not less. Rare back-mutation to non-streptomycin-requiring forms occurred. A recent paper by Kushnick, Randles, Gray and Birkeland deals with variants (mutants) of *E. coli*, *P. aeruginosa* and *B. subtilis* requiring streptomycin.² All mutants showed this growth stimulation when glucose was present in the medium. In the case of *P. aeruginosa* sodium formate could replace the glucose. The case of *B. subtilis* was unusual in that the streptomycin-requiring mutants grew only under anaerobic conditions. Many papers in the literature^{3,4} demonstrate that the antibacterial action of streptomycin is greatly reduced by anaerobic conditions.

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² Kushnick, T., Randles, C. I., Gray, C. T., and Birkeland, J. M., *Science*, 1947, **100**, 587.

³ Donovick, R., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 224.

⁴ Bondi, A., Deitz, C. C., and Spaulding, E. H., *Science*, 1946, **103**, 399.

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6	80	Very few	Small	1280	Frequent	Undifferentiated
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8	40	Very few	"	160	"	"
9	80	"	"	40	"	"
10	20	Frequent	Undifferentiated	40	"	"
11	160	None	Small	20	None	Small
12	160	"	Undifferentiated	20	"	"
13	40	"	"	20	"	"
14	10	"	"	10	"	Large
15	10	"	"	40	Very few	Undifferentiated
				10	"	"

* Concentration of streptomycin in units per ml on which colony grew and from which transfer was made.

† Frequency of colonies on control streptomycin-free medium; i.e., reversion to parent type no longer requiring streptomycin as essential metabolite.

TABLE IV.
Relation of Stimulatory and Antibacterial Activity of Different Active Substances.

	Stimulatory activity on D57 _M <i>E. coli</i>	Antibacterial activity on D57 <i>E. coli</i>
Streptomycin pool III	1.0	1.0
Streptomycin A	1.4	1.9
Dihydrostreptomycin A	1.4	2.1
Streptobiosamine HCl	0.06	0.07
N-Acetyl streptomycin A	0.18	
Streptomycin B	0.7	0.54

"water extract," containing 3.9% inositol, from Dr. H. E. Carter (see footnote to Table III) were inactive, but a brain lipositol labeled "ether extract," containing 6.3% inositol, from Dr. H. E. Carter was slightly active at 20 γ to 160 γ but not in lower concentration.

The relative growth promoting capacities on mutant D57_M of the substances labeled active in Table III are shown in Table IV. These activities have been referred to that of the streptomycin pool III as unity and are to be regarded as only approximate since the dilutions tested varied from each other by 2-fold. Also shown are the antibacterial activities of these same substances on the parent strain D57 again referred to the activity of pool III as unity. These values are accurate to within $\pm 5\%$.

It will be noted that the relative values of the growth stimulatory activity agree remarkably well with the antibacterial values of the same substances on the parent strain. The activity of the N-acetyl streptomycin A corresponds to the 15% of streptomycin A which this sample was known to contain. In the case of the sample of streptobiosamine HCl it is not certain whether the activities found are due to a residuum (3.5%) of streptomycin A or whether the compound itself has

activity. This is being investigated further. It is of interest that the relative activities of streptomycin B show such good agreement. This would suggest that the same factors may act to decrease the stimulatory activity of streptomycin B relative to A as act to decrease its antibacterial activity on the parent culture. One can perhaps visualize the extra mannose moiety as interfering with the activities of the rest of the molecule.

Summary. Streptomycin-requiring mutants were obtained from 2 of 3 strains of meningococci and 3 of 4 strains of *E. coli*. In the case of 2 *E. coli* strains such mutants grew readily on a synthetic medium in the presence of streptomycin. Such mutants appear more stable when isolated from low rather than from high streptomycin concentrations. In the case of the *E. coli* mutants, they exhibit optimal growth at concentrations from 20 to 80 units/ml of a commercial streptomycin or of pure streptomycin A. Growth of the mutants can be promoted by pure preparations of streptomycin A, dihydrostreptomycin A, and streptomycin B and the relative stimulatory activities of these compounds for the mutant agrees closely with the relative antibacterial activities against the parent strain.

My thanks are due to Mr. John Oskay for technical assistance in carrying out these experiments.

TABLE III.
Substances* Active as Essential Metabolites for *E. coli* Mutant D 57_M.

Active	Inactive
Streptomycin A	D-glucosamine HCl
Streptomycin B	Streptodine sulfate ¹¹
Dihydrostreptomycin A	Pneumococcus polysaccharides ¹²
N-Acetyl streptomycin A †, ‡	<i>K. pneumoniae</i> polysaccharides ¹²
Streptobiosamine HCl †, ‡, §	Streptomine ¹¹ dihydrochloride
Lipositol §	L-(+)-Arabinose
	N methyl-L-glucosaminic acid ¹²

* My thanks are due to Dr. Oskar Wintersteiner and Dr. Josef Fried of the Division of Organic Chemistry, The Squibb Institute for Medical Research, for all the streptomycin and derivative samples; to Dr. H. E. Carter, Department of Chemistry, University of Illinois, and Dr. D. W. Woolley, The Rockefeller Institute for Medical Research, for samples of lipositol; to Dr. J. W. Palmer, Biological Laboratories, E. R. Squibb and Sons, for the polysaccharides of the pneumococcus types and *K. pneumoniae* type B; and to Dr. M. Heidelberger, Department of Medicine, Columbia University, for the polysaccharides of *K. pneumoniae* types A and C.

† Contains 15% streptomycin A.

‡ Activity probably due to residual streptomycin A (see text).

§ Some preparations showed slight activity (see text).

|| Types 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 14, 18, 19.

¶ Types A, B, and C.

cation to occur for longer than 72 hours back-mutation has been rare and growth has rarely appeared in the control (streptomycin-free) medium within this time. When cultures are held for longer periods, mutants not requiring the antibiotic gradually emerge. For all of the routine passage and selection of mutants, in this work with the synthetic medium, a streptomycin pool designated III has been used. This has the following characteristics: streptomycin A 65%, streptomycin B 30%, other maltol producing material 5%, total streptomycin 64%, inert material 33%, minimal inhibiting concentration (M.I.C.) for *K. pneumoniae* (in the standard biological assay)⁸ 0.103 γ /ml, M. I. C. inhibiting *E. coli* (D57 parent strain) 0.155 γ , *K. pneumoniae*/

E. coli ratio 0.67, streptomycin potency 354 units/mg.

Using this strain D57_M the capacity of many different substances to replace the streptomycin pool III in the medium as essential metabolite has been investigated. The results are shown, in summary, in Table III.

One notes that streptidine and streptomine are inactive. Also inactive are the polysaccharide fractions of pneumococci and *K. pneumoniae*. The latter inactivity is particularly to be noted. We also received and tested 3 samples of polysaccharides of *M. tuberculosis* strain H37 kindly supplied by Dr. M. Heidelberger of the Department of Medicine, Columbia University. These represent the high rotating (fraction C), low rotating (fraction B_{2a}) and serologically inert (fraction B_{2c}) fractions described elsewhere.¹³ All were negative.

As far as the active substances are concerned it is to be noted that lipositol showed very little activity and that such activity as was obtained was not found with every sample. Thus a sample from soybean provided by Dr. D. W. Woolley (see footnote to Table III) was slightly active at 20 γ and 40 γ per ml but not in lower or higher concentration. A soybean lipositol and a brain lipositol labeled

⁸ Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

⁹ Fried, J., and Stavely, H. E., to be published.

¹⁰ Kuehl, F. A., Jr., Flynn, E. H., Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 2096; Fried, J., Walz, D. E., and Wintersteiner, O., *J. Am. Chem. Soc.*, 1946, **68**, 2746.

¹¹ Carter, H. E., et al., *Science*, 1946, **103**, 53; Fried, J., Boyack, J. A., and Wintersteiner, O., *J. Biol. Chem.*, 1946, **162**, 391; Peck, R. L., et al., *J. Am. Chem. Soc.*, 1946, **68**, 29, 776.

¹² Kuehl, F. A., Jr., Flynn, E. H., Holly, F. W., Mozingo, R., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 536; Wolfson, M. L., Thompson, A., and Hooper, R. I., *J. Am. Chem. Soc.*, 1946, **68**, 2343.

¹³ Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

and included in any study of an outbreak of a vesicular disease of cattle or swine.

The methods used are still under investigation in a study of the complement-fixation reaction in foot-and-mouth disease. Refinement of technic, however, should lead only to greater delicacy in the detection of immunological differences, a goal greatly desired in respect of foot-and-mouth disease and perhaps not without significance for vesicular stomatitis.

Methods. Some details of the strains of virus used are given since other experiments with the same strains have been published by American workers.

Vesicular Stomatitis (V.S.) type Indiana Strain USCB, received from Dr. Schoening of the U. S. Bureau of Animal Industry in 1944. It was derived from bovine material from an outbreak in Colorado. It is believed that this strain is identical with that described by Shahan, Frank, and Mott⁹ as Ind C. It has been maintained at this Institute by serial passage in cattle, and the material used in the experiments below was of the 8th passage. Epithelium was collected from the tongue of an infected steer on 10th June, 1947, cut up, and dried over sulphuric acid and, later, P₂O₅. It was stored in sealed ampoules at 4°C.

V.S. type New Jersey Strain USKP, received as above, and derived from an outbreak in Kansas City, Mo. Later described by the workers cited above as NJM. Two batches of material were used, one of the 3rd passage stored frozen at -20°C from 25th May, 1947, and the other from the 5th passage, dried and stored as the Indiana type material. Guinea pig material of the two type strains was also included, in each case as the 13th serial passage of these strains in guinea pigs, stored as frozen guinea pig pads at -20°C.

Foot-and-Mouth Disease (F. & M.) type Vallee "O" Strain ASJ, an Argentine strain of bovine origin. Passaged in cattle at Pirbright from 1944 till 10 April, 1946. Bovine tongue epithelium was collected and stored

in 50/50 glycerine and M/25 phosphate buffer at pH 7.6.

F. & M. type Vallee "A" Strain 119, a bovine strain, originating from an outbreak in Great Britain in 1932, and since given 68 serial passages in cattle. Material collected and stored as for "O" type, from 14th October, 1947.

F. & M. type Waldmann "C" Strain 149. Also a bovine field strain, recovered in Great Britain in 1934, and since given 33 serial passages at this Institute. Epithelium was collected on 10th November, 1947, and stored frozen.

Three strains of foot-and-mouth disease, passaged in guinea pigs, were also included in the work. They were: Strain 1, "O" type, passaged at Pirbright since 1924, Strain GB, "A" type passaged since 1929, and Strain GC, "C" type, passaged since 1933.

For use in the complement-fixation test, an approximately 1/10 suspension (calculated on wet weight) was prepared, in 0.9% NaCl for V.S. and in M/25 phosphate buffer for F. & M. The suspensions were prepared from 4-18 hours before use, and then centrifuged at 2,000 r.p.m. for 10 minutes immediately before test. The specific sera were all obtained from guinea pigs which had passed through the disease. In the case of V.S. the infecting strains were those described above, while for F. & M. the strains used were the stock guinea pig strains used in this Institute. The guinea pigs used for V.S. (Indiana) serum had received a subsequent intramuscular inoculation of virus before use as serum donors and those for F. & M. (O, A, and C) serum, a course of 4 inoculations by the same route. The F. & M. sera were lyophilized, but the V.S. sera were stored as fluid sera at 4°C. The complement was a stock lyophilized guinea pig serum prepared in batches for use in routine complement-fixation tests in F. & M. The hemolytic system was obtained from a reputable supplier of biological products.

Details of the test methods will be published in full when the data on F. & M. are presented. Briefly, the procedure is to carry out an abbreviated titration of complement

⁹ Shahan, M. S., Frank, A. H., and Mott, L. O., 1946, *loc. cit.*

Vesicular Stomatitis and Foot-and-Mouth Disease Differentiation by Complement-Fixation.

J. B. BROOKSBY. (Introduced by R. E. Shope.)

From the Research Institute of the Foot-and-Mouth Disease Research Committee, Pirbright, Surrey, England.

It is generally admitted that the differential diagnosis of vesicular stomatitis and foot-and-mouth disease on clinical grounds alone is virtually impossible. Methods of differentiating between the two diseases which have been suggested include: (a) determination of the particle size of the infective agent (Galloway and Elford¹); (b) determination of infectivity for the developing egg by the chorioallantoic route (Burnet and Galloway,² Galloway and Elford³); (c) study of the pH stability range of the infective agent (Pyl and Klenk,⁴ Pyl,⁵ Reppin, and Pyl⁶); (d) tests of susceptibility of several species of animal (e.g., horses, cattle, swine, sheep, guinea pigs) by a variety of routes; (e) cross-immunity tests in experimental animals (Wagener).⁷

In addition may be included epidemiological criteria, such as spreading power and degree of generalization of the disease in infected animals. These are of little use in countries such as the United States of America and Great Britain, where the first object of policy is to limit the possible spread of the infective vesicular diseases of animals by the slaughter of infected stock, and destruction of all material likely to harbour the viruses.

All the methods listed above depend, in the last analysis, on the susceptibility of the test animals, and the virulence of the material

used. It is therefore doubtful if too great reliance can be placed on any one method. For example, if, in a test of species susceptibility, one or two horses do not react to inoculation with an unknown virus, which has however infected a similar number of cattle and swine, a diagnosis of foot-and-mouth disease cannot be given with confidence. Similarly, the criterion tentatively adduced by Shahan, Frank, and Mott,⁸ depending on the intramuscular inoculation of cattle, has already been found by the same workers to be unreliable, in that an animal inoculated with the virus of vesicular stomatitis has reacted, while observations at this Institute with some strains of the virus of foot-and-mouth disease show that the result of intramuscular inoculation of foot-and-mouth disease virus is not always regular.

In these circumstances, any data which can be gathered rapidly, on an infective agent causing vesicular lesions in cattle and swine, are of great value. The virtues of an *in vitro* test, in respect of speed as much as of economy, are especially attractive. These are the considerations which have stimulated publication of the present paper. Of necessity, since the work has been done in a country where the virus of vesicular stomatitis has not so far been identified in the field, the observations are based on strains which have been passaged in the laboratory; but the well-defined results obtained—better than those usually encountered under comparable conditions with the virus of foot-and-mouth disease—suggest that the method could be of great value with material from field outbreaks. The results presented below support the view that complement-fixation should be added to the list of methods of differential diagnosis,

¹ Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14**, 400.

² Burnet, F. M., and Galloway, I. A., *Brit. J. Exp. Path.*, 1934, **15**, 105.

³ Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1935, **16**, 588.

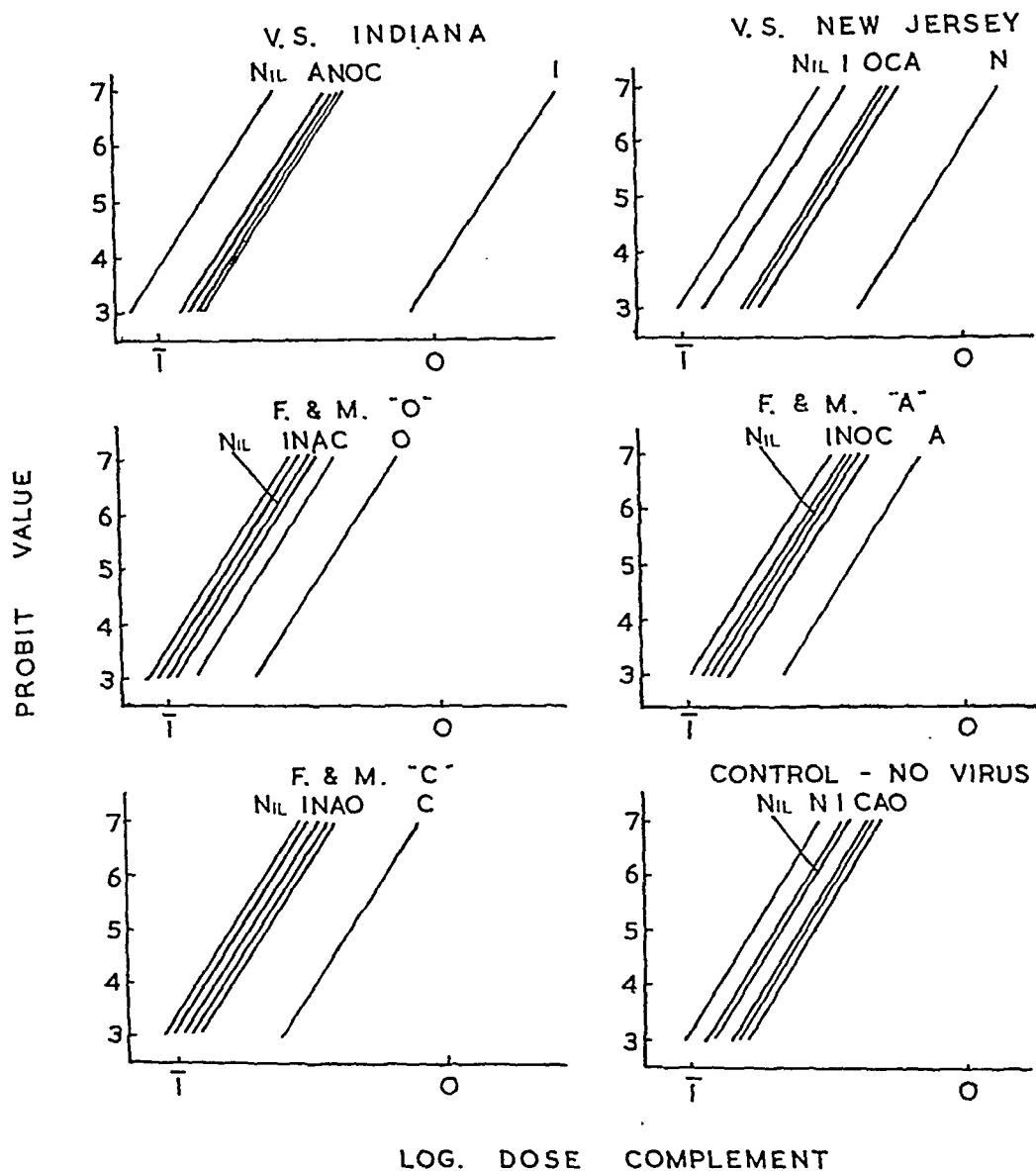
⁴ Pyl, G., and Klenk, L., *Zbl. Bakt. Abt.*, 1933, 1, Orig. **128**, 161.

⁵ Pyl, G., *Hoppe-Seyler's Z.*, 1934, **226**, 18.

⁶ Reppin, K., and Pyl, G., *Arch. wiss. prakt. Tierh.*, 1934, **68**, 183.

⁷ Wagener, K., *Arch. wiss. prakt. Tierh.*, 1933, **66**, 173, 301, 363.

⁸ Shahan, M. S., Frank, A. H., and Mott, L. O., *J. Am. Vet. Med. Assn.*, 1946, **108**, 5.



LOG. DOSE COMPLEMENT

FIG. 1.

Complement fixation in vesicular stomatitis and foot-and-mouth disease. Antisera used are indicated thus: I = V.S. Indiana; N = V.S. New Jersey; O = F. & M. "O"; A = F. & M. "A"; C = F. & M. "C"; Nil = No antiserum (antigen control).

tained from guinea pigs convalescent from the disease, whereas the Indiana anti-serum came from guinea pigs which had had an additional inoculation with virus, by the intramuscular route. That the difference between the results was dependent on the sera was suggested by an experiment where guinea pig

and bovine virus of the two types were tested against the same two sera. The results here showed that the degree of fixation recorded with each serum ran parallel in the presence of the different antigens.

The F. & M. type-immune sera were all from guinea pigs which had been hyper-

TABLE I.
Type-specific Fixation of Complement in Vesicular Stomatitis and Foot-and-Mouth Disease.

Antigen	Type	Antiserum					
		Vesicular stomatitis		Foot-and-mouth disease			Nil
		Indiana	New Jersey	"O"	"A"	"C"	
Vesicular stomatitis	{ Indiana	1.44	.23	.24	.22	.24	.14
	{ New Jersey	.21	.74	.30	.32	.30	.17
Foot-and-mouth disease	{ "O"	.15	.17	.37	.19	.23	.18
	{ "A"	.18	.20	.23	.39	.24	.21
	{ "C"	.17	.19	.21	.20	.44	.16
Nil	—	.20	.16	.27	.26	.26	.21

N.B. The figure in each case represents the amount of complement, as ml of 1/25 guinea pig serum, necessary for 50% hemolysis.

in the presence of the various reagents, combined, and with suitable controls. The doses of complement are arranged in a logarithmic series, and the end-point used is 50% hemolysis, calculated from the readings obtained with the centrifugalized tests on a photo-electric absorptiometer ("Spekker" by Hilger, London). The amounts of the various reagents used are (1) virus, 0.2 ml of 1/10 suspension; (2) serum, 0.4 ml of 1/8 dilution in 0.9% NaCl; (3) complement, 1 ml of a dilution of guinea pig serum in 0.9% NaCl, calculated to give the appropriate dose of a series, e.g., 0.2, 0.3, 0.45, 0.67, and 1.0 ml of a 1/25 dilution. Where a reagent is omitted, the volume is made up with 0.9% NaCl.

Fixation of complement is allowed to take place in a water bath at 37°C for 30 minutes. The hemolytic system is then added (0.5 ml of 3.3% cells with hemolytic included) and incubation continued for a further 30 minutes. The tubes are then removed from the water bath, and centrifuged at 1,500 r.p.m. for 10 minutes. Readings are made directly on the tubes, held in a special mount. From these readings, the per cent hemolysis for each tube is obtained from a graph, and hence the amount of complement for the 50% hemolysis end-point is calculated.

Alternatively, the results may be presented graphically, plotting the per cent hemolysis against the log. dose of complement. Titration of complement alone and in the presence of virus and specific sera gives a sigmoid curve in such graphs, and as it is more convenient

to deal with straight lines, the transformation evolved by Gaddum¹⁰ and Bliss¹¹ is used. The values for per cent hemolysis are converted to probit values, using the tables of Fisher and Yates,¹² and these values are plotted against the log dose of complement. The probit value 5.0 corresponds to 50% hemolysis and the end-points are easily obtained from these graphs.

Results. Type specific complement-fixation has been obtained using virus from cattle or guinea pigs in foot-and-mouth disease and from both these sources and also from infected eggs in vesicular stomatitis. Since the results are substantially the same with different antigens, the results of a representative test in which virus of bovine origin was employed is shown in Table I, and, graphically in Fig. 1. The fixation of complement by the homologous virus-serum mixture is demonstrated by the increased amount of complement necessary for 50% hemolysis. In the figure, this is shown by the shift to the right of the appropriate graph.

The result with V.S. Indiana virus is particularly clear-cut. The degree of fixation exceeds by two- or three-fold that hitherto encountered in F. & M. disease, under optimum conditions. The poorer fixation with V.S. New Jersey virus may be explained by the fact that the homologous serum was ob-

¹⁰ Gaddum, J. H., No. 183, Spec. Rep. Ser., Med. Res. Coun., H. M. Stationery Office, London, 1933.

¹¹ Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

¹² Fisher, R. A., and Yates, F., *Statistical Tables*, 2nd edit., Edinburgh, Oliver & Boyd, 1943.

Proceedings

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No. 3

SECTION MEETINGS

CLEVELAND, OHIO	
Western Reserve University	February 13, 1948
DIS. DIST. OF COLUMBIA	
George Washington University	February 5, 1948
ILLINOIS	
Northwestern University	February 10, 1948
MINNESOTA	
University of Minnesota	February 11, 1948
NEW YORK	
Rockefeller Institute	February 25, 1948
ROCKY MOUNTAIN	
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SOUTHERN	
Birmingham, Ala.	January 16, 1948
SOUTHERN CALIFORNIA	
University of Southern California	January 29, 1948
WESTERN NEW YORK	
University of Rochester	February 21, 1948

16270

Skeletal Growth in Pyridoxine Deficient Mice.*

RUTH SILBERBERG AND BARNET M. LEVY.

From the Snodgrass Laboratory, City Hospital, the Barnard Free Skin and Cancer Hospital, and the Department of Oral Pathology, Washington University, School of Dentistry, St. Louis, Mo.

The present investigation deals with the histological changes in the long bones associated with the arrest of skeletal growth in pyridoxine deficient mice.

Material and Methods. Twenty-eight male and female mice of strain C57 black, 23 days old, were used. Fourteen animals were placed on a synthetic diet complete except for the lack of pyridoxine (Group I). Fourteen animals were placed on a synthetic diet

complete except for pyridoxine but containing 54% casein (Group II). Animals of corresponding strain and age, fed a stock diet (Purina Laboratory Chow), used in previous experiments served as normal controls. The rations were composed as follows:

	Group I	Group II (Pyridoxine free, high protein, low sucrose)
Pyridoxine (free)	68 g.	30 g.
Sucrose	18 "	54 "
Vit. test casein	10 "	10 "
Vegetable oil	4 "	4 "
U.S.P. salt mix. No. 12	4 "	4 "

* Aided by a grant of the Committee on Scientific Research of the American Medical Association and the U. S. Public Health Service.

immunized, and yet the difference in the amount of complement necessary for 50% hemolysis, in the presence of homologous as compared with heterologous serum and virus, is only about two-fold. The quantitative difference between V.S. and F. & M. in respect of the complement fixing properties may be correlated with the behavior with neutralizing antibodies. Studies on neutralization of the virus of V.S. by its antisera show that the degree of neutralization recorded for typical sera greatly exceed the corresponding values for F. & M. and its antisera. Thus a 1:10 dilution of V.S. serum may neutralize 10^7 I.D.₅₀ of virus (Skinner¹³) while no similar dilution of F. & M. serum has yet been found to neutralize as much as 10^3 I.D.₅₀.

Discussion. It must be emphasized that the results recorded were obtained with material of only 2 strains of the virus of vesicular stomatitis. Confirmation using other strains is greatly to be desired, in view of the diffi-

culties which have attended the examination of a large proportion of strains of the virus of foot-and-mouth disease from field outbreaks. The bovine V.S. material used was epithelium about 4-6 months old, and 2 of the samples had been dried. It is not known how fresh material, collected, perhaps, at a time when the virus was not fully active, would behave in the test. Nevertheless, in view of the clear-cut results recorded here, an optimistic view of the value of the test is probably justified, and the addition of complement fixation to the armory of tests to which a virus of this group can be submitted would seem to be well worth while.

Summary. (1) The fixation of complement by vesicular stomatitis virus and its antiserum is described. (2) This fixation is type specific for the 2 strains of vesicular stomatitis virus of Indiana and New Jersey type available. (3) There is no cross-fixation with any of the three types of the virus of foot-and-mouth disease.

¹³ Skinner, H. H., personal communication, 1947.

Expenditures

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Sales

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Experimental Error

~~Time 25~~

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SECRET

CLEVELAND OHIO

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site. However, after
favorable influence of
became noticeable.
the upper end of the
ons in width as com-



Fig. 3.

The upper end of the tibia of a rat 65 days old, kept deficient for 4 weeks, and the epiphyseal cartilage. The epiphysal bone has a typical appearance. The epiphysal bone is well developed and shows no signs of replacement.

Special Agent in Charge

From the Swedish Laboratory
the Department of Civil Pathology

The present investigation deals with histological changes in the liver associated with the administration of pyridoxine deficient mice.

Material and Methods. Two hundred and female mice of strains C57BL/6J and old, were used. Fourteen mice were placed on a special diet for the lack of sixteen animal.

* Aided by a grant from the
National Institute of the Deaf and
the U. S. Public Health Service.

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¹³ Skinner, H. H., personal communication, 1947.

young rats fed a pyridoxine deficient diet,³ changes in the growth zones were comparable to those of underfed young guinea pigs kept on a quantitatively restricted but qualitatively balanced diet. To some extent this is true for the cartilage of mice also. However, the cartilage cells as well as the osteoblasts responded more unfavorably to pyridoxine deficiency than to general underfeeding, and the formation of bone was more reduced than in any of our previous experiments. This difference might indicate

a specific effect of the pyridoxine deficiency, but it may be a manifestation of the more marked sensitivity of the mouse to the deficiency as compared to that of the rat.^{5,6}

Feeding a high protein diet accentuated the effects of pyridoxine deficiency inasmuch as growth of cartilage and bone was more inhibited than in pyridoxine deficient mice receiving a diet with regular casein content. These findings are in agreement with previous observations on the greater requirements for pyridoxine in animals fed high protein diets.

³ Antopol, W., and Unna, K., *Arch. Path.*, 1942, 3, 241.

⁴ Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, 30, 675.

⁵ Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, 1944, 5, 207.

⁶ Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, 1945, 157, 551.

16271

Experimental Fort Bragg Fever (Pretibial Fever) in Chimpanzees.*

JOSEPH L. MELNICK AND JOHN R. PAUL. (Introduced by Francis G. Blake.)

From the Section of Preventive Medicine, Yale University School of Medicine, New Haven.

The chimpanzee (*Pan Satyrus*) has been used extensively in experimental work on poliomyelitis^{1,2} and to a lesser degree in work on the common cold,³ but the use of this animal as an experimental host for the reproduction of human infectious disease is a field deserving further exploration. Particularly would this seem to apply to the virus field and to diseases which have readily discernible cutaneous manifestations. We have recently made attempts to induce in these animals 3 human virus diseases: sandfly (Phlebotomus or pappataci) fever, dengue fever, and Fort Bragg (pretibial) fever. This paper will be concerned only with a description of our experiments with the latter disease. Results with the other two diseases will be described in another paper.⁴

Fort Bragg fever was first described by Daniels and Grennan,⁵ under the term *pretibial fever*. It is a disease characterized by an acute fever of approximately 5 days' duration, frontal and postorbital aching, splenomegaly, and often with a patchy erythematous rash on the pretibial regions.[†] Respiratory manifestations are minimal and not persistent. Cases of this disease have appeared in groups during the summer months of 1942, 1943, and 1944 on the military reservation at Fort Bragg, N. C. The manner in which it spreads among humans has not been determined.

² Melnick, J. L., and Horstmann, D. M., *J. Exp. Med.*, 1947, 85, 257.

³ Dochez, A. R., Shibley, G. S., and Mills, K. C., *J. Exp. Med.*, 1930, 52, 701.

⁴ Paul, J. R., Melnick, J. L., and Sabin, A. B., to be published.

⁵ Daniels, V. H., and Grennan, H. A., *J. M. M. A.*, 1943, 122, 361.

[†] The rash described by Daniels and Grennan is a prevalent feature that is cellular and has been designated this disease a *developmental fever*.
The rash is magnified.

* This investigation was conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

¹ Bodian, D., and Howe, H. A., *J. Exp. Med.*, 1947, 81, 255.



FIG. 4.

High power photograph of a bony trabecula of the same animal as in Fig. 1. The spicule is thick and is covered by a continuous layer of large osteoblasts.

pared with 90 microns in mice kept on the deficient diet with regular protein content. The cells were smaller and fewer than in the latter group, and the matrix was more hyalinized. These differences were accentuated after 8 weeks and manifested themselves chiefly in the absence of hypertrophic cartilage cells and in even more advanced hyalinization of the matrix.

Resumption of growth after refeeding was slower in the group receiving the high protein diet. A noteworthy enlargement of the epiphyseal discs was not seen until after 5 days of realimentation. Even after 10 days, the cartilage cells were still smaller than ordinarily, although the individual cartilage cell row contained the usual number of cells. Mitotic proliferation of cartilage cells was less prominent indicating a comparatively slow rate of growth. In the bone, plugs of degenerated cartilage and hard smearable ground substance were still present. Osteoblasts were rather scanty. They had not

yet completely regained their epithelioid shape.

Summary and Conclusions. In pyridoxine deficient young mice, growth of cartilage and formation of bone were inhibited and finally ceased. Upon refeeding the complete diet, recovery was noticeable after 3 days and was still in progress after 14 days. The growth processes were quickly restored with mitoses present in the germinal layer of the cartilage as well as in the osteoblasts.

The effects of pyridoxine deficiency on cartilage resemble those seen in pantothenic acid deficient mice,¹ whereas the disturbance of ossification was not unlike that observed in riboflavin deficiency.² The specificity of the skeletal changes may thus be questioned.



FIG. 5.

High power photograph of 2 bony trabeculae of a female mouse of strain C57, 12 weeks of age, and fed a pyridoxine-deficient diet for 8 weeks. The spicules are thin and covered only here and there by spindle-shaped cells. Osteoblasts are not seen. The bone marrow is atrophic. Same magnification as No. 4.

¹ Levy, B. M., and Silberberg, M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 380.

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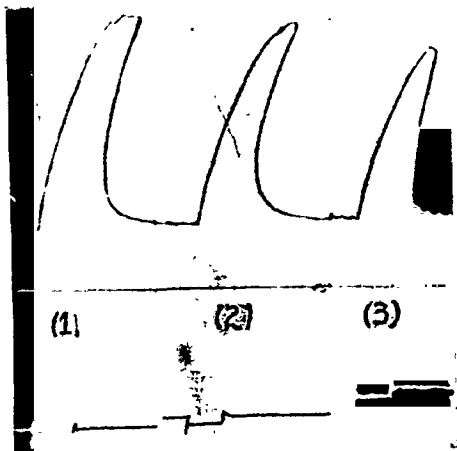


FIG. 1.

Kymograms showing the effect of stimulation of the contra-lateral sciatic nerve in causing reflex contraction of (1) the normal (left) soleus, (2) the transplanted (right) soleus, (3) the normal (left) tibialis posterior.

minimal degree of after-discharge. It is apparent, nevertheless, that the characteristic of the contraction is more nearly that of pale muscle than of red.

In Table I are compared the analyses for iron and myohemoglobin in normal soleus, transplanted soleus, and normal tibialis pos-

TABLE I.
Comparison of Myohemoglobin and Iron Contents in Normal Red, Transplanted Red, and Normal White Muscles.

All muscle weights corrected to 5 g. All values represent averages in mg per 5 g muscle of the number of determinations indicated in parentheses.

Muscle	Iron content	Myohemoglobin content
Tibialis posterior (2)	32.50	10.00
Transplanted soleus (3)	34.00	10.29
Normal soleus (3)	53.49	15.74

terior muscles. It is evident from these results that the alteration in function of the red muscle has resulted in an iron and myohemoglobin content very similar to that of normal pale muscle and far below that of normal red muscle.

Summary. Conversion of red muscle to pale muscle can be accomplished by changing the position of the tendon of insertion to that of a white muscle. In thus assuming the function of a pale synergistic muscle, the red muscle in turn assumes the properties of pale muscle as evidenced by similar types of reflexly induced contractions and by similar myohemoglobin and iron contents. Acknowledgment is due Mrs. G. W. Wyatt for aid in the chemical determinations.

16273

Sulfhydryl and Disulfide Content of Normal and Arsenic-Resistant Trypanosomes.*

STEWART C. HARVEY.[†] (Introduced by E. M. K. Geiling.)

The sulfhydryl group has been regarded as a more or less specific receptor for tervalent arsenicals since it was postulated by Voegtlin¹ that arsenicals are bound through the formation of dithioarsenite complexes. It was further suggested by him² that differences in the equilibrium position of the glutathione system or in the absolute quantity of glutathione in sub-strains of a given strain of trypanosomes were responsible for the phenomenon of ar-

senic-resistance, the resistant strain presumably having a greater sulfhydryl reserve with which to detoxify the arsenical. Since then, the concept of the arsenic receptor has been broadened to include the fixed sulfhydryl groups of proteins,^{3,4} the importance of which has been emphasized by the work of Barron

¹ Voegtlin, C., Dyer, H. A., and Leonard, C. S., *U. S. Pub. Health Rep.*, 1923, **38**, 1882.

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* Supported by a grant-in-aid from the U. S. Public Health Service.

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by a short febrile period with viremia and by the appearance of neutralizing antibodies in the serum a few weeks after the infection.

16272

Conversion of Red Muscle to Pale Muscle.*

L. M. N. BACH. (Introduced by H. S. Mayerson.)

From the Department of Physiology, Tulane University School of Medicine, New Orleans.

It has long been taught that the function of myohemoglobin in red muscle is its function as an oxygen reservoir for the sustained contractions so typical of this kind of muscle.¹ Variations in myohemoglobin content were noted by Whipple² who correlated the content of this pigment with the age, state of health, and amount of activity engaged in by dogs. McClintock, Hines, and Jordan³ observed that increased activity of muscle would lead to an increase in myohemoglobin content. In order to test the validity of the concept that myoglobin content and type of activity are correlated, the experiments described below were carried out.

Methods. Large, mature rabbits were selected for these experiments. Six such animals were used and each was anesthetized with nembutal. Under aseptic conditions, the tendon of the right soleus muscle was severed and the central end of this tendon sutured to the peripheral end of the severed tendon of the synergistic pale tibialis posterior muscle. By causing the red soleus to utilize the insertion of the pale tibialis muscle, it was hoped to convert the soleus to a pale muscle. The ankle was fixed with an orthoplast bandage in such a way that the foot was kept partially flexed for a period of 2 weeks at the end of which time the bandage was removed. The animals were then sacri-

ficed after a period of 6 months. At sacrifice, the animal was placed under nembutal anesthesia and the normal tibialis and soleus of the left leg and the transplanted soleus of the right leg were arranged for recording. All tendon sutures were found to have been successful. The contractions of each of these extensor muscles were elicited as a part of a crossed extensor reflex elicited by stimulation of the contralateral sciatic nerve.

After the recordings were made, the animal was perfused with saline, and the muscle was excised and weighed. Hemoglobin of muscle was determined by the acid hematin method according to Whipple⁴ and iron determinations were simultaneously made by the method of Wong.⁵

Results A comparison of reflexly induced tetanic contractions is shown in Fig. 1. This typical result illustrates the fact that excitation with stimuli of equal strength (10 volts), equal frequency (60 c.p.s.), and equal duration (1 sec.) result in a prolonged and maximal degree of contraction, marked by considerable after-discharge in the normal soleus and a very short, lesser degree of tetanus with a conspicuous lack of after-discharge in the case of the normal tibialis posterior. The transplanted soleus, on the other hand, exhibits a reflexly induced contraction which is remarkably similar to the contraction of the pale tibialis muscle. There remain, however, some remnants of red muscle contraction characteristics as evidenced by the greater extent of contraction with the pale muscle and by the existence of a

* Aided by a grant from the David Trautman Schwartz Research Fund of the Tulane School of Medicine.

¹ Millikan, G., *Physiol. Ref.*, 1939, **19**, 509.

² Whipple, G. H., *Physiol.*, 1926, **76**.

³ McClintock, J. T., Hines, H. M., and Jordan, D. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 139.

⁴ Whipple, G. H., *Physiol.*, 1926, **76**, 693.

⁵ Wong, S. Y., *J. Chem.*, 1928, **77**, 409.

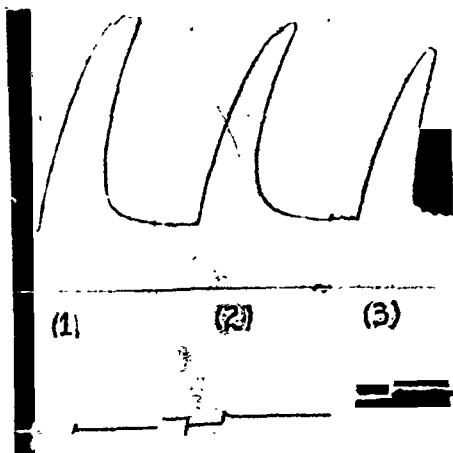


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Methods. Large, mature rabbits were selected for these experiments. Six such animals were used and each was anesthetized with nembutal. Under aseptic conditions, the tendon of the right soleus muscle was severed and the central end of this tendon sutured to the peripheral end of the severed tendon of the synergistic pale tibialis posterior muscle. By causing the red soleus to utilize the insertion of the pale tibialis muscle, it was hoped to convert the soleus to a pale muscle. The ankle was fixed with an orthoplast bandage in such a way that the foot was kept partially flexed for a period of 2 weeks at the end of which time the bandage was removed. The animals were then sacri-

ficed after a period of 6 months. At sacrifice, the animal was placed under nembutal anesthesia and the normal tibialis and soleus of the left leg and the transplanted soleus of the right leg were arranged for recording. All tendon sutures were found to have been successful. The contractions of each of these extensor muscles were elicited as a part of a crossed extensor reflex elicited by stimulation of the contralateral sciatic nerve.

After the recordings were made, the animal was perfused with saline, and the muscle was excised and weighed. Hemoglobin of muscle was determined by the acid hematin method according to Whipple⁴ and iron determinations were simultaneously made by the method of Wong.⁵

Results A comparison of reflexly induced tetanic contractions is shown in Fig. 1. This typical result illustrates the fact that excitation with stimuli of equal strength (10 volts), equal frequency (60 c.p.s.), and equal duration (1 sec.) result in a prolonged and maximal degree of contraction, marked by considerable after-discharge in the normal soleus and a very short, lesser degree of tetanus with a conspicuous lack of after-discharge in the case of the normal tibialis posterior. The transplanted soleus, on the other hand, exhibits a reflexly induced contraction which is remarkably similar to the contraction of the pale tibialis muscle. There remain, however, some remnants of red muscle contraction characteristics as evidenced by the greater extent of contraction than with the pale muscle and by the existence of a

* Aided by a grant from the David Trautman Schwartz Research Fund of the Tulane School of Medicine.

1 Millikan, G., *Physiol. Ref.*, 1939, **19**, 509.

2 Whipple, G. H., *Physiol.*, 1926, **76**.

3 McClintock, J. T., Hines, H. M., and Jordan, D. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 139.

4 Whipple, G. H., *Physiol.*, 1926, **76**, 693.

5 Wong, S. Y., *J. Chem.*, 1928, **77**, 409.

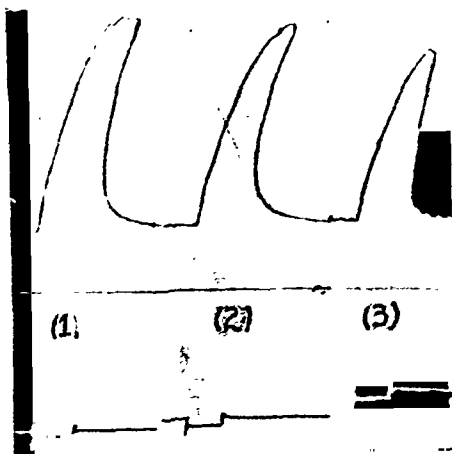


FIG. 1.

Kymograms showing the effect of stimulation of the contra-lateral sciatic nerve in causing reflex contraction of (1) the normal (left) soleus, (2) the transplanted (right) soleus, (3) the normal (left) tibialis posterior.

minimal degree of after-discharge. It is apparent, nevertheless, that the characteristic of the contraction is more nearly that of pale muscle than of red.

In Table I are compared the analyses for iron and myohemoglobin in normal soleus, transplanted soleus, and normal tibialis pos-

TABLE I.
Comparison of Myohemoglobin and Iron Contents in Normal Red, Transplanted Red, and Normal White Muscles.

All muscle weights corrected to 5 g. All values represent averages in mg per 5 g muscle of the number of determinations indicated in parentheses.

Muscle	Iron content	Myohemoglobin content
Tibialis posterior (2)	32.50	10.00
Transplanted soleus (3)	34.00	10.29
Normal soleus (3)	53.49	15.74

terior muscles. It is evident from these results that the alteration in function of the red muscle has resulted in an iron and myohemoglobin content very similar to that of normal pale muscle and far below that of normal red muscle.

Summary. Conversion of red muscle to pale muscle can be accomplished by changing the position of the tendon of insertion to that of a white muscle. In thus assuming the function of a pale synergistic muscle, the red muscle in turn assumes the properties of pale muscle as evidenced by similar types of reflexly induced contractions and by similar myohemoglobin and iron contents. Acknowledgment is due Mrs. G. W. Wyatt for aid in the chemical determinations.

16273

Sulfhydryl and Disulfide Content of Normal and Arsenic-Resistant Trypanosomes.*

STEWART C. HARVEY.[†] (Introduced by E. M. K. Geiling.)

The sulfhydryl group has been regarded as a more or less specific receptor for tervalent arsenicals since it was postulated by Voegtlin¹ that arsenicals are bound through the formation of dithioarsenite complexes. It was further suggested by him² that differences in the equilibrium position of the glutathione system or in the absolute quantity of glutathione in sub-strains of a given strain of trypanosomes were responsible for the phenomenon of ar-

senic-resistance, the resistant strain presumably having a greater sulfhydryl reserve with which to detoxify the arsenical. Since then, the concept of the arsenic receptor has been broadened to include the fixed sulfhydryl groups of proteins,^{3,4} the importance of which has been emphasized by the work of Barron

¹ Voegtlin, C., Dyer, H. A., and Leonard, G. S., *U. S. Pub. Health Rep.*, 1923, **38**, 1882.

² Voegtlin, C., Dyer, H. A., and Miller, D. W., *J. Pharm. and Exp. Therap.*, 1924, **23**, 55.

³ Voegtlin, C., and Rosenthal, S. M., *J. Pharm. and Exp. Therap.*, 1930, **39**, 347.

* Supported by a grant-in-aid from the U. S. Public Health Service.

[†] John Jacob Abel Fellow Pharmacology.

by a short febrile period with viremia and by the appearance of neutralizing antibodies in the serum a few weeks after the infection.

16272

Conversion of Red Muscle to Pale Muscle.*

L. M. N. BACH. (Introduced by H. S. Mayerson.)

From the Department of Physiology, Tulane University School of Medicine, New Orleans.

It has long been taught that the function of myohemoglobin in red muscle is its function as an oxygen reservoir for the sustained contractions so typical of this kind of muscle.¹ Variations in myohemoglobin content were noted by Whipple² who correlated the content of this pigment with the age, state of health, and amount of activity engaged in by dogs. McClintock, Hines, and Jordan³ observed that increased activity of muscle would lead to an increase in myohemoglobin content. In order to test the validity of the concept that myoglobin content and type of activity are correlated, the experiments described below were carried out.

Methods. Large, mature rabbits were selected for these experiments. Six such animals were used and each was anesthetized with nembutal. Under aseptic conditions, the tendon of the right soleus muscle was severed and the central end of this tendon sutured to the peripheral end of the severed tendon of the synergistic pale tibialis posterior muscle. By causing the red soleus to utilize the insertion of the pale tibialis muscle, it was hoped to convert the soleus to a pale muscle. The ankle was fixed with an orthoplast bandage in such a way that the foot was kept partially flexed for a period of 2 weeks at the end of which time the bandage was removed. The animals were then sacri-

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After the recordings were made, the animal was perfused with saline, and the muscle was excised and weighed. Hemoglobin of muscle was determined by the acid hematin method according to Whipple⁴ and iron determinations were simultaneously made by the method of Wong.⁵

Results A comparison of reflexly induced tetanic contractions is shown in Fig. 1. This typical result illustrates the fact that excitation with stimuli of equal strength (10 volts), equal frequency (60 c.p.s.), and equal duration (1 sec.) result in a prolonged and maximal degree of contraction, marked by considerable after-discharge in the normal soleus and a very short, lesser degree of tetanus with a conspicuous lack of after-discharge in the case of the normal tibialis posterior. The transplanted soleus, on the other hand, exhibits a reflexly induced contraction which is remarkably similar to the contraction of the pale tibialis muscle. There remain, however, some remnants of red muscle contraction characteristics as evidenced by the greater extent of contraction than with the pale muscle and by the existence of a

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³ McClintock, J. T., Hines, H. M., and Jordan, D. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 139.

⁴ Whipple, G. H., *Physiol.*, 1926, **76**, 693.

⁵ Wong, S. Y., *Chem.*, 1928, **77**, 409.

in the medium and to the salt concentration.

3. The effect of salt is exerted not upon the streptomycin itself but upon the medium,

which in turn influences the growth of the organism.

16277

Effect of Organic Acids on Streptomycin Activity.*†

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From the Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University.

The antibacterial potency of streptomycin is still largely measured by its inhibiting effect upon the growth of certain bacteria in artificial culture media, under given conditions of cultivation. The activity of the antibiotic thus measured is influenced by a great many factors. Chief among these is the presence of certain organic and inorganic constituents in the medium,¹ the presence of certain reducing substances,^{2,3} the nature of the test organism, its age and number of viable cells used.⁴ In addition to these, the presence of certain organic acids that may be formed as intermediary metabolic products in the nutrition of various bacteria may also affect the activity of streptomycin. A study of the last phenomenon forms the subject of this report.

Escherichia coli ATTC 9637 was used as the test organism. Its growth in various media was determined by means of a Cenco-Sheard-Sanford Photometer. Immediately after inoculation, the culture tubes gave a

reading of zero turbidity. The final turbidity was expressed in terms of per cent of light absorption.

The organic acids used in these studies were neutralized to pH 7.0, sterilized by filtration through Mandler candles, and added to the medium to give a final concentration of 1%. The streptomycin solution was sterilized by heating at 60°C for 30 minutes. The basic nutrient broth used in these experiments contained 0.5% peptone and 0.3% meat extract.

The influence of different organic acids as compared to that of sugars and glycerol upon streptomycin activity was at first determined (Table I). The growth of *E. coli* in nutrient broth is usually inhibited by 1 µg of streptomycin per ml. When pyruvic or fumaric

TABLE I.
Effect of Various Carbon Sources in Medium upon Streptomycin Activity on *E. coli*.

Carbon source 1%	Turbidity*			
	4 hr		21 hr	
	Streptomycin, 10 µg/ml		Streptomycin, 10 µg/ml	
	0	+	0	+
Glucose	—	—	41	0
Lactose	14	0	36	0
Pyruvic acid	23	13	67	31
Fumaric acid	14	12	53	30
Formic acid	0	0	15	12
Succinic acid	8	6	30	7
Glycerol	8	0	42	0
Maleic acid	10	6	40	12
Malonic acid	8	6	31	6
Glycerophosphoric acid	7	0	33	0
Acetic acid	6	0	34	0
Propionic acid	5	0	30	0
Lactic acid	9	0	52	0

* Absorption of light in per cent.

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Commonwealth Fund of New York.

¹ Green, S. R., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **67**, 281.

² Geiger, W. B., Green, S. R., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 157.

³ Donovan, R., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 224.

⁴ Lenert, T. K., and Hobby, G. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 235, 242.

TABLE IV.
Combined Effect of NaCl and Peptones on Streptomycin Activity in Glucose Media.
Cultures incubated for 22 hours at 28°C.

Peptone source	NaCl, 0.5%	Meat extract, 0.3%	Turbidity	
			Streptomycin, 0	10 µg/ml, +
Peptone 327393	0	0	27	0
" 327393	+	0	19	19
" 327393	0	+	45	30
" 327393	+	+	47	38
" 23368	0	0	22	0
" 23368	+	0	23	20
" 23368	0	+	43	0
" 23368	+	+	41	37
" 383611	0	0	25	0
" 383611	+	0	22	21
" 383611	0	+	43	27
" 383611	+	+	44	35
Tryptone	0	0	30	0
"	+	+	37	29
"	0	+	45	0
"	+	+	48	38

TABLE V.
Effect of Salt upon Streptomycin Activity in Agar Media.
Cup method used; zone of inhibition measured in mm.*

Concentration of NaCl, %	Salt added to agar. Streptomycin, µg/ml			Salt added to streptomycin solution. Streptomycin, 10 µg/ml
	5	10	50	
0	13.0	14.0	18.5	17.0
0.5	14.0	16.0	20.0	18.0
1.0	12.0	14.0	19.0	18.1
3.0	9.0	9.0	14.0	19.0

* Diameter of cup 9 mm.

antibiotic, thus proving that the streptomycin was not destroyed.

The salt phenomenon was shown to be effective not only for *E. coli* but also for *Bacillus subtilis* and *Proteus vulgaris*. Furthermore, other salts, such as magnesium chloride, sodium nitrate, and sodium sulfate, in *M/3* concentrations, also inhibited the antibacterial activity of streptomycin. These results are in accord with those obtained by other investigators. The salt effect was not neutralized by the addition of phosphate buffer or of serum to the medium.

When sodium chloride was added to nutrient agar, and the activity of streptomycin tested by the agar diffusion or cup method, it was found (Table V) that 0.5% salt actually caused an increase in the width of the zone of inhibition due to streptomycin; the

addition of 3% salt to the agar resulted, however, in a marked reduction in the size of the zone. When salt was added to the streptomycin solution before it was placed in the cup, there was no neutralizing action, but an actual stimulating effect upon the potency of streptomycin.

These results indicate that the effect of salt is due not to a modification of the streptomycin but to a change in the conditions in the medium which influence the growth of the test organisms.

Summary. 1. The potency of streptomycin is greatly influenced by the composition of the medium, the salt concentration being most significant.

2. The inhibiting effect of glucose upon streptomycin activity is due to the specific nature of the organic nitrogenous compounds

TABLE II.
Effect of Different Concentrations of Streptomycin upon Growth of *E. coli* in Presence of Pyruvic or Fumaric Acids.

Carbon source 1%	Streptomycin, $\mu\text{g/ml}$	Turbidity			
		5 hr	8 hr	17 hr	22 hr
Pyruvic acid	0	14	19	50	48
	10	7	14	33	34
	25	2	5	23	27
	50	0	0	0	2
	100	0	0	0	0
Fumaric acid	0	10	25	49	49
	10	9	20	40	41
	25	6	16	29	32
	50	1	3	15	16
	100	0	0	0	0

TABLE III.
Influence of Nitrogen Sources upon Effect of Organic Acids and Other Carbon Compounds upon Streptomycin Activity.*

Additional material	Casein hydrolysate		Asparagine Streptomycin, 10 $\mu\text{g/ml}$		Tryptone	
	0	+	0	+	0	+
None	20	0	2	0	22	0
Glucose	38	0	2	0	39	0
Lactose	33	0	0	0	30	0
Formic acid	4	3	4	0	13	6
Maleic acid	29	22	5	5	21	10
Malonic acid	44	10	11	0	21	4
Malic acid	51	15	16	0	40	18
Succinic acid	38	10	12	0	30	10
Pyruvic acid	48	37	50	2	42	40
Glycerol	37	0	2	0	29	0
Fumaric acid	35	22	17	2	36	19
Lactic acid	57	0	9	0	36	0

* *E. coli* used as test organism; incubation 20 hours at 28°C.

acid was added to the medium good growth was obtained even in the presence of 10 $\mu\text{g/ml}$ of the antibiotic. Succinic, formic, maleic, and malonic acids also exerted a certain degree of protection upon the bacteria against the antibacterial action of streptomycin. Lactose, glycerol, sodium glycerophosphate, lactate, and glucose had, however, no effect upon streptomycin activity; the same was true of propionic and acetic acids.

The pyruvate and fumarate media in which *E. coli* grew in the presence of streptomycin were tested, by the agar cup method, for streptomycin concentration. The antibiotic was not reduced in potency. The possible development of bacterial strains resistant to streptomycin was also determined; the culture was found to remain sensitive to 2 $\mu\text{g/ml}$ of streptomycin.

A study was made next of the minimum concentration of streptomycin necessary to inhibit the growth of *E. coli* in the presence of pyruvate and fumarate. Even in the presence of 50 $\mu\text{g/ml}$ of streptomycin good growth was obtained in the peptone broth to which fumarate was added; there was no growth, however, with 100 $\mu\text{g/ml}$ streptomycin (Table II). The effect of pyruvate was similar to that of fumarate, although quantitatively somewhat different. When the concentration of the fumarate was increased to 3%, growth occurred even in the presence of 150 $\mu\text{g/ml}$ of streptomycin but none with 200 $\mu\text{g/ml}$.

The addition of organic acids and sugars to tryptone or casein hydrolysate broth gave results similar to those obtained with the peptone broth (Table III). When asparagine

was used as a source of nitrogen, slight growth of *E. coli* occurred in the presence of streptomycin with maleic acid, and a trace of growth with pyruvic and fumaric acids; the other carbon sources did not antagonize the action of streptomycin upon the growth of *E. coli*. This indicated the possibility that the complex nitrogenous materials contained some substance which was necessary for the utilization of the pyruvate and fumarate in the presence of streptomycin; this substance could not be derived from asparagine.

In an effort to elucidate the possible nature of the substance which favored the neutralizing effect of pyruvate and which was absent in asparagine, yeast extract and a vitamin mixture containing B₁, B₂, B₆, calcium pantothenate, folic acid, nicotinamide, para-aminobenzoic acid, and cocarboxylase were added to asparagine-pyruvate broth. Only the yeast extract proved effective in allowing the growth of *E. coli* in the presence of streptomycin (Table IV).

The neutralizing effect of pyruvate and fumarate upon streptomycin was tested with other bacteria, including another strain of *E. coli* (No. 2), *Acrobacter aerogenes*, *Proteus vulgaris*, and *Staphylococcus aureus*. The action of pyruvate and fumarate upon streptomycin activity was found to vary with the organism (Table V). It was most marked with *P. vulgaris* and *E. coli*, and least with *A. aerogenes* and *S. aureus*. Similar variations were obtained with other organisms. *Mycobacterium tuberculosis* No. 607 showed some inhibition by fumarate with low concentrations of streptomycin and none with higher concentrations; the pyruvate showed no effect at all for this organism.

The results tend to show that certain organic acids have a definite neutralizing or antagonistic effect upon the antibacterial properties of streptomycin only as regards certain organisms. The salts of the dicarboxylic acids (fumaric, succinic, malic, and maleic), as well as pyruvic and formic acids, were most effective; they supported the growth of *E. coli* in the presence of bactericidal concentrations of streptomycin. The salts of acetic, lactic, and propionic acids, as well as sugars, such as glucose and lactose,

TABLE IV.
Effect of B Vitamins and Yeast Extract upon
Antibacterial Activity of Streptomycin in
Asparagine-Pyruvate Broth.
Asparagine 2.5 mg/ml, pyruvate 10 mg/ml.

Supplementary additions to broth	Mg/ml	Turbidity after 25 hrs Streptomycin, 10 µg/ml	
		0	+
Broth alone*		13	0
Pyruvate control		28	28
Yeast extract	2.5	51	17
" "	1.25	50	6
" "	0.63	52	8
Vitamin mixture		8	0

* No pyruvate.

did not influence the inhibiting action of streptomycin upon *E. coli*.

The presence of pyruvic and dicarboxylic acids does not result in the destruction of the antibiotic, since the concentration of streptomycin in the medium remained close to the theoretical. These organic acids do not favor the development of strains of bacteria resistant to the action of streptomycin. No irreversible combination is produced between the antibiotic and the organic acid. The only explanation for this effect may be looked for in the susceptibility of a specific enzyme system to the action of streptomycin, whereby it may be replaced, at least in the metabolism of certain bacteria, by another system in the presence of pyruvic or fumaric acid. That the effect of streptomycin upon susceptible bacteria may be due to interference with intermediary metabolism has been suggested.⁶

It may also be of interest to note here that the oxidation of benzoic acid by *Mycobacterium tuberculosis* No. 607 was inhibited by 10 µg/ml streptomycin, whereas even 100 µg had no effect upon this oxidation process by a resistant strain of this organism. On the other hand, 100 µg streptomycin had no effect upon the oxidation of pyruvic acid by the normal strain.⁷

The possibility that streptomycin interferes with the synthesis of amino acids, proteins, or carbohydrates and that the organic

⁵ Geiger, W. B., *Arch. Biochem.*, 1947,

⁶ Strauss, E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 97.

⁷ Bernheim, F., and Fitzgerald, R. J., *Science*, 1947, **105**, 435.

TABLE V.
Effect of Pyruvate and Fumarate upon Antibacterial Activity of Streptomycin.
Incubation 17 hours at 28°C.

Carbon source	<i>E. coli</i>		<i>A. aerogenes</i>		<i>P. vulgaris</i>		<i>S. aureus</i>	
	0*	+	0	+	0	+	0	+
None	15	0	18	0	4	0	24	0
Pyruvic acid	36	29	40	4	36	26	44	13
Fumaric acid	40	23	46	6	47	42	22	6
Glucose	37	0	56	0	14	0	44	0

* 0 = no streptomycin; + = 4 μ g/ml streptomycin.

acids may counteract the antibiotic effect by acting as essential metabolites in the synthesis of these compounds has been suggested.⁵ Certain preliminary results obtained in oxidation experiments, by the use of the Thunberg and Warburg techniques, indicated that streptomycin does not affect metabolic reactions measured in terms of oxygen uptake or dehydrogenase activity; rather it appeared that streptomycin affects a synthetic process, and the effect on respiration is only a secondary phenomenon.

Summary. The addition of 1% pyruvate and fumarate to nutrient broth supported the growth of *E. coli* in the presence of 10 μ g/ml of streptomycin. When the concentration of the acids was increased to 3%, growth took place even in the presence of 150 μ g/ml of streptomycin. The streptomycin was not de-

stroyed. The test bacteria growing in the presence of streptomycin and of the organic acids did not become resistant to streptomycin.

Salts of succinic, formic, malic, and maleic acids also exerted some antagonistic effect upon streptomycin. Lactose, as well as lactic, acetic, and propionic acids, glycerol, glycerophosphate, and glucose had no effect upon the growth-inhibiting action of streptomycin on *E. coli*.

Comparison of the antagonistic effect of pyruvic and fumaric acids against the streptomycin action upon various bacteria, showed considerable variation. *E. coli* and *P. vulgaris* were largely protected by the organic acids against the action of streptomycin, whereas *A. aerogenes* and *S. aureus* were only slightly affected.

16278

Urinary Excretion of 17-Ketosteroids by Normal Young Men During Starvation.*

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There are only a few reports indicating the effect of starvation on the urinary excretion

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of 17-ketosteroids. In most of these, owing

Society of Friends, Philadelphia; the Mennonites Central Committee, Akron, Pa.; the John and Mary R. Markle Foundation, New York; the Sugar Research Foundation, New York; the National Dairy Council operating on behalf of the American Dairy Association, Chicago; and the Home Missions Board of the Unitarian Society, Boston. We wish to thank Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N.J., for generous supplies of androsterone.

to a number of complicating factors, it is impossible to assess the influence of the restricted food intake on the urinary excretion.

Dingemanse and her associates¹ found a marked decrease in the concentration of 17-ketosteroids in the urine of the inhabitants of the Netherlands during the German occupation. As they point out, although the content per unit volume was lower during the war than afterwards, the absolute amounts excreted in the two periods may not have been very different since the urine volume during the occupation was 1.5 to 2 times normal.

Salter *et al.*² noted a slight decrease in the mean urinary excretion of 17-ketosteroids among 48 repatriated American prisoners-of-war from the Pacific theater. All of these men had gynecomastia at one time or another. The biochemical manifestations of gynecomastia in these ex-P.O.W.'s were said to have been very similar to those observed in similar cases among civilians where the breast hypertrophy and tumor was presumably due to causes other than malnutrition. The papers by the Yale workers give the impression that the decreased 17-ketosteroid excretion is not a manifestation of starvation *per se*.

Chou and Wang³ reported that the excretion of male sex hormones (as determined by the same general technique used in the 17-ketosteroid analysis) was markedly reduced in malnourished patients in proportion to the degree of undernutrition. However, factors other than starvation must have been operative in these cases since their well-nourished patients showed a rate of excretion of male sex hormones only 42% of the mean for their normal subjects.

Methods. The amount of total neutral 17-ketosteroids in the urine was determined by the micro-method of Miller and Mickelsen.⁴

¹ Dingemanse, E., Huis in't Veld, L. G., and de Laat, B. M., *J. Clin. Endocrinol.*, 1946, **6**, 535.

² Salter, W. T., Klatskin, G., and Humm, F. D., *Am. J. Med. Sci.*, 1947, **213**, 31.

³ Chou, C. Y., and Wang, C. W., *Chinese J. Physiol.*, 1939, **14**, 151.

⁴ Miller, E. v. O., and Mickelsen, O., to be published.

This involved the simultaneous hydrolysis of the urine with hydrochloric acid and extraction with carbon tetrachloride. After a series of washings with potassium hydroxide, hydrochloric acid and water, the carbon tetrachloride extract was evaporated to dryness *in vacuo*. The residue was dissolved in ethyl alcohol and then subjected to the usual Zimmermann reaction. In all cases duplicates were carried through from the beginning of the procedure and repeated when they failed to agree within 4%. All urine specimens were collected in bottles containing glacial acetic acid as a preservative. The analyses were started within 24 hours of the time of collection, or the sample was stored at -20°C until the analysis could be made.

Semi-starvation experiment. Thirty-four normal young men between 19 and 32 years of age voluntarily submitted to a semi-starvation experiment. During the 6-month period when they were maintained on an average daily intake of 1600 calories, they lost 24% of their original body weight. The diet simulated that available to the inhabitants of north-central Europe and was relatively adequate in all determinable factors other than calories and protein. Before the experiment started, the subjects were put through various physical and psychological tests in order to exclude those who deviated from normal. Other details of this experiment have been described elsewhere.⁵

A random group of 10 of these men was chosen for the study of the 17-ketosteroid excretion. The semi-starvation phase of the experiment was preceded by a 3-months control period. No urine samples were collected for these special analyses at that time. However, control samples were collected from some of the men on the 57th week of rehabilitation when the subjects were back to normal in all respects. Additional control urinary excretion values from a large group of similar young men were available for comparative purposes.

Urine samples were collected for the deter-

⁵ Henschel, A., Mickelsen, O., Taylor, H. L., and Keys, A., *Am. J. Physiol.*, 1947, **150**, 170; Keys, A., *J. Am. Diet. Assn.*, 1946, **22**, 582.

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	0*	+	0	+	0	+	0	+
None	15	0	18	0	4	0	24	0
Pyruvic acid	36	29	40	4	36	26	44	13
Fumaric acid	40	23	46	6	47	42	22	6
Glucose	37	0	56	0	14	0	44	0

* 0 = no streptomycin; + = 4 µg/ml streptomycin.

acids may counteract the antibiotic effect by acting as essential metabolites in the synthesis of these compounds has been suggested.⁵ Certain preliminary results obtained in oxidation experiments, by the use of the Thunberg and Warburg techniques, indicated that streptomycin does not affect metabolic reactions measured in terms of oxygen uptake or dehydrogenase activity; rather it appeared that streptomycin affects a synthetic process, and the effect on respiration is only a secondary phenomenon.

Summary. The addition of 1% pyruvate and fumarate to nutrient broth supported the growth of *E. coli* in the presence of 10 µg/ml of streptomycin. When the concentration of the acids was increased to 3%, growth took place even in the presence of 150 µg/ml of streptomycin. The streptomycin was not de-

stroyed. The test bacteria growing in the presence of streptomycin and of the organic acids did not become resistant to streptomycin.

Salts of succinic, formic, malic, and maleic acids also exerted some antagonistic effect upon streptomycin. Lactose, as well as lactic, acetic, and propionic acids, glycerol, glycerophosphate, and glucose had no effect upon the growth-inhibiting action of streptomycin on *E. coli*.

Comparison of the antagonistic effect of pyruvic and fumaric acids against the streptomycin action upon various bacteria, showed considerable variation. *E. coli* and *P. vulgaris* were largely protected by the organic acids against the action of streptomycin, whereas *A. aerogenes* and *S. aureus* were only slightly affected.

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Urinary Excretion of 17-Ketosteroids by Normal Young Men During Starvation.*

ERMA V. O. MILLER, OLAF MICKELSEN, AND ANCEL KEYS.

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There are only a few reports indicating the effect of starvation on the urinary excretion

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Regents of the University of Minnesota. This contract was subsequently transferred to the Office of the Surgeon General, U. S. Army. Financial assistance was also provided by the Brethren Service Committee, Elgin, Ill.; the Service Committee of the

of 17-ketosteroids. In most of these, owing

Society of Friends, Philadelphia; the Mennonites Central Committee, Akron, Pa.; the John and Mary R. Markle Foundation, New York; the Sugar Research Foundation, New York; the National Dairy Council operating on behalf of the American Dairy Association, Chicago; and the Home Missions Board of the Unitarian Society, Boston. We wish to thank Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N.J., for generous supplies of androsterone.

TABLE II.

The Urinary Excretion of 17-Ketosteroids During Acute Starvation. All values for ketosteroid excretion are expressed as mg of androsterone per 24 hours. The mean urine volumes as cc per 24 hours are given. The standard deviations are given with the means. The decrease in ketosteroid excretion observed on the fourth day of starvation is expressed as a percentage of the control value (% Max. Deccr.)

Subject	Control period	3rd day starvn.	4th day starvn.	% Max. Deccr.
B	11.7	5.2	4.7	59.8
A	12.4	5.7	2.9	76.6
M	11.3	4.1	3.4	69.9
D	9.1	4.3	3.0	67.0
Pa	9.9	3.6	2.7	72.7
Pe	13.8	4.9	3.7	73.2
C	10.9	3.8	3.2	70.6
H	12.9	4.7	4.7	63.6
E	12.8	5.2	4.3	66.4
W	9.3	2.7	2.3	75.3
S	9.6	3.3	2.6	72.9
R	13.3	4.6	5.5	58.6
Mean	11.41 \pm 1.66	4.34 \pm 0.88	3.58 \pm 1.00	68.9
Urine vols.	902 \pm 245	745 \pm 258	813 \pm 445	

food intake was raised, the ketosteroid excretion increased. By R6, the mean excretion level for the 8 men examined then was 11.9 mg. The ketosteroid excretion returned practically to normal in spite of the fact that in even those subjects receiving the largest amount of food, body weight restoration amounted to a maximum of 18% of that lost during starvation with the values for most of the men below 7%. The recovery of physical fitness at this time was also very low. A subsequent year on an unlimited diet produced only a negligible increase in the 17-ketosteroid excretion. Subject 130 whose excretion was below normal at R6 was the one whose food intake during this period was the lowest. His recovery, on the basis of such factors as restoration of body weight, improvement of physical performance, etc., was considerably less than that of the other subjects. This subject was unusual in that his excretion level at R6 was so very much lower than the value at S24. In 2 cases (1 and 26) the excretion at R6 was much greater than at R57. There is no apparent explanation for these findings.

Results—acute starvation experiment. The 3 days of acute starvation produced a marked reduction in the 17-ketosteroid excretion of all 12 subjects (Table II). The average excretion during the control period was 11.41 ± 1.66 mg per 24 hours whereas that for the third day of the experiment was 4.34 ± 0.88

and that for the fourth day was 3.58 ± 1.00 . In practically every case, the 24-hour urinary excretion was below the lower normal limits. On the fourth day of starvation, the ketosteroid output was still lower than that of the preceding day in all but 2 cases.

By far the greatest part of the reduction in the urinary excretion occurred by the third day of starvation. The decrease by that time averaged 62% of the control values whereas the total reduction was 69%. The reduction of the ketosteroid excretion ranged from 59 to 77%. There was no relation between the magnitude of the reduction and the control ketosteroid level.

The rate of excretion of ketosteroids in these two types of starvation was not influenced by the urine volume. In the semi-starvation experiment during the entire starvation phase the daily urine volume was about twice the control value. In the acute starvation experiment, on the other hand, the urine volume was markedly reduced during the starvation period.

Discussion. Both acute and semi-starvation with hard work produced marked reductions in the rate of total, neutral 17-ketosteroid excretion, the influence of acute starvation being much greater than that of semi-starvation. Any attempt to explain this change must involve a consideration of the functions influenced by each of the experiments. The plane of metabolism cannot be

TABLE I.

Urinary Excretion of 17-Ketosteroids During Semi-starvation and Subsequent Rehabilitation. All values are expressed as mg of androsterone per 24 hours. The mean urine volumes as cc per 24 hrs are given. The standard deviations are given with the means.

Subject	S8	S24	R6	R57
1	10.8	10.2	17.6	12.6
9	10.8	9.8	12.8	15.0
12	3.3	8.7	10.9	11.7
104	7.7	6.9	9.8	11.2
122	7.0	6.4	9.6	10.3
Mean	7.92 \pm 3.12	8.40 \pm 1.70	12.14 \pm 3.31	12.16 \pm 1.79
23		4.7	10.7	13.0
26		7.7	18.2	10.1
109		8.0		11.0
130		11.5	5.8	17.5
232	8.6	8.8		
Mean (all subjects)	8.03 \pm 2.80	8.27 \pm 1.99	11.92 \pm 4.18	12.49 \pm 2.42
Urine vol. (all subjects)	1848 \pm 759	3009 \pm 1794	3838 \pm 925	1380 \pm 383

mination of the ketosteroid excretion at the following times: (1) the eighth week of starvation (S8) when the men had lost an average of 13% of their original body weight, (2) at the twenty-fourth week of semi-starvation (S24) when they had lost 24% of their original body weight, (3) at the sixth week of rehabilitation (R6) when the men had regained some of their lost weight and (4) at the 57th week of rehabilitation (R57) when they were back to normal in all respects.

Acute starvation experiment. Twelve normal young men were subjected to a 4½-day period when they received no food. Water was the only substance permitted by mouth during this period and it was unrestricted. Before and after this, these men were the subjects of a long-time experiment during which they received diets controlled as far as calories and thiamine were concerned. Throughout the starvation period, the physical activity of the subjects was maintained by standardized walking on a motor-driven treadmill. The work was of such intensity that a calculated caloric deficit of approximately 5000 calories per day was produced. The training factor involved in this high plane of activity was cancelled out since these men had done the same amount of work for a week or more preceding the fast.

Urine samples for the estimation of the 17-ketosteroid excretion were collected on the third and fourth days of the fast. The control samples were secured some months later.

These values agreed with those secured a number of months preceding the experiment. The validity of these control samples has been independently proven by the constancy of the 24-hour urinary excretion of total, neutral 17-ketosteroids over periods as long as a year or more.^{6,7}

Results—semi-starvation experiment. The excretion of total, neutral 17-ketosteroids for the 5 men studied at S8 averaged 7.9 mg per 24 hours (Table I). This is considerably below the mean excretion level of 11.3 mg observed for normal young men in this laboratory⁷ as well as the 12.2 mg for these men at the 57th week of rehabilitation. Each subject at S8 showed a lower excretion than that during the recovery period. In fact subject 12 showed an excretion level considerably below any observed by us in normal young men, while the values for the rest of the subjects were in the lower normal range.

At S24, the 17-ketosteroid excretion of the above group showed no essential change except for subject 12 whose value increased considerably. The values for the other men were essentially the same as those at S8. Five additional men studied at S24 showed the same decrease in 17-ketosteroid excretion as the preceding group.

During the rehabilitation period when the

⁶ Miller, E. v. O., Mickelsen, O., and Keys, A., to be published.

⁷ Miller, E. v. O., Mickelsen, O., and Keys, A., *Fed. Proc.*, 1947, 6, 279.

TABLE I.
Toxoplasma Neutralization Tests in Rabbits.

Rabbit No.	Toxoplasma strain	Toxoplasma mixed with	Dilution of mouse brain suspension			
			1:10	1:50	1:500	1:5000
203	Bk	Tyrode's sol.	++++	+++	+	—
	Bk	Mother's serum	+	+	—	—
230	S	Tyrode's sol.	++	+	+	—
	S	Mother's serum	+	—	—	—
232	Bk	Tyrode's sol.	++++	++++	++	—
	Bk	Immune monkey serum	++	+	—	—
	S	Tyrode's sol.	++++	++++	+	—
	S	Immune monkey serum	++	+	—	—

++++ Strong swelling and necrosis.

+++ Strong swelling without necrosis.

++ Moderate swelling without necrosis.

+ Slight swelling.

— No reaction.

TABLE II.
Cross Immunity Between "Bk" and "S" Strains of Toxoplasma.

Rabbit No.	First inoculation			Second inoculation				
	Date	Strain	Reaction	Date	Strain	Reaction	Strain	Reaction
250	Oct. 14	S	++++	Dec. 1	S	+	Bk	+
202	Nov. 12	S	++++	1	S	++	Bk	++
213	7	Bk	++++	1	S	++	Bk	+
214	7	Bk	++++	1	S	++	Bk	++

Significance of + to ++++ as in Table I.

twitchings of arms and legs, rotations of the eye-balls, developing hydrocephalus, neck stiffness, opisthotonos, muscle rigidity and scissor stand of the legs; soon convulsions appeared.

Dr. C. D. Binkhorst, who examined the eyes, diagnosed anterior chorioretinitis, bilateral peripheral ablatio retinae, atrophy of the papillae and strabismus convergens. Dr. Berkyens, who performed a ventriculography, reported displacement and enlargement of the lateral ventricles, especially of the right one which was displaced to the left, $\frac{1}{2}$ inch beyond the median line.

The cerebrospinal fluid, obtained by ventricular puncture, contained xanthochrome and tryptophane. The reactions of Nonne and Pandy were strongly positive, and there were 196 cells per cu mm. In the smear of the centrifuged sediment of this fluid (August 7) we found many half-moon-shaped parasites resembling toxoplasma. They were not found in the blood, urine and bone-marrow.

On August 11, fresh cerebrospinal fluid was inoculated intracerebrally into 10 mice; 8 of them died after 7 to 25 (average 15) days, and toxoplasma could be demonstrated in the brains. In serial transmissions, the length of the incubation period decreased and from the third intracerebral passage 100% of the mice died after 5 to 6 days.

Guinea pigs inoculated intracerebrally with fresh cerebrospinal fluid from the patient, remained healthy, but those inoculated with toxoplasma-containing mouse brain suspensions died after 12 to 15 days.

Dr. A. B. Sabin kindly provided us with a toxoplasma strain of human origin (called by us strain S) for comparison with ours (strain Bk). We carried out a number of neutralization experiments (rabbit skin tests as described by Sabin¹) with both strains, which were tested against serum of the infant's mother and against lyophilized toxoplasma immune monkey serum. The latter

¹ Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, 51, 6.

the primary factor since in the acute starvation subjects this was at a very high level whereas in the semi-starvation subjects it was sharply reduced.

There is the possibility that the decrease in the ketosteroid excretion may be explained on the basis of a change in the activity of the testes. During the semi-starvation experiment there was a reduction in sexual functions as revealed by alteration in libido and in the morphology and physiological responses of the sperm collected at that time. Similar studies were not made in the acute starvation experiment, but here, too, at least libido was decreased. Other factors than the testes may be considered in explaining the decrease in ketosteroid excretion observed in acute and semi-starvation. This is apparent when one considers the marked differences in the ketosteroid excretion in these two experiments. Changes in the function and activity of the adrenals under these conditions can only be surmised at the present time.

The hard physical work in the acute starvation experiment had no influence on the ketosteroid excretion since the same work was done in the control period. Furthermore, in other experiments where the same work was done while the men were in caloric

balance, there was no change in the ketosteroid excretion. Besides the physical "stress" in the acute starvation, both experiments were associated with considerable mental "stress" but in spite of this the ketosteroid excretion in each experiment decreased. This is contrary to other reports in the literature which have emphasized the increased ketosteroid excretion in "stressful" situations.⁵

Summary. The urinary excretion of total, neutral 17-ketosteroids was determined in 10 young men who, over a 6-months period, lost 24% of their original body weight. The reduction in ketosteroid excretion was 30% of the control values.

Twelve young men performed hard physical work during 4 days when they received no food. By the fourth day the urinary excretion of 17-ketosteroids was only 31% of the control value. The ketosteroid excretion was not influenced by the hard work since the subjects were trained to it before the start of the fast.

⁵ Pincus, G., and Hoagland, H., *J. Aviation Med.*, 1943, **14**, 173; Hoagland, H., *Science*, 1944, **100**, 63; Pincus, G., in *Recent Progress in Hormone Research*, Acad. Press, Inc., New York, 1947

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Isolation of Toxoplasma from Cerebrospinal Fluid of a Living Infant in Holland.

J. WINSSER, J. D. VERLINDE, P. H. VAN THIEL, J. DAVEL, AND P. VAN DER ELST.
(Introduced by Albert B. Sabin.)

From the Institute of Preventive Medicine, the Laboratory of Parasitology, University of Leiden, and the Southern Hospital, Rotterdam, Holland.

Recent observations raise the supposition that toxoplasmosis is not infrequent in Europe. In most of the cases, the parasitological confirmation of the clinical diagnosis has been made post mortem by the demonstration of toxoplasma in brain sections. Toxoplasma have been demonstrated rarely in the cerebrospinal fluid during life of the

patient by microscopic examination and animal inoculation. In the summer of 1947, however, we succeeded in this.

Our patient, the second child of apparently healthy parents, was a 6-weeks-old boy who did not show any signs of illness during the first few weeks of life. On August 7, the infant was admitted to the hospital, showing

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Of 16 rabbits used for the skin test, 8 died 9 to 12 days after the inoculation. At autopsy an enlarged spleen with white foci, containing toxoplasma, was found regularly. Of the 8 surviving animals 4 were re-inoculated intracutaneously with 0.2 cc of a 10% mouse brain suspension at 4 separate spots, in order to test cross-immunity. Skin lesions of moderate size developed, but without necrosis. The results of the cross-immunity experiments are shown in Table II.

There was no striking difference in the skin reactions produced by the strains used. Only one of the animals died, but at autopsy no lesions specific for toxoplasmosis could be found, so that an intercurrent death is suspected. Evidence of cross-immunity has been given by this experiment.

As no differences in behavior, morphology,

virulence, cross-neutralization and cross-immunity between the strains Bk and S can be observed, we believe that they are closely related or even may be identical.

The infant died on September 21. Treatment with sulphathiazole was not successful. At autopsy Dr. H. E. Schornagel found toxoplasma-containing mononuclear infiltrations in the meninges, the walls of the ventricles and the cerebral tissue, and foci of necrosis with many mononuclear cells and extracellular parasites. In the subcutaneous tissue, the psoas muscle and the diaphragm, some toxoplasma-containing mononuclear infiltrations were found.

Summary. This is a report of a case of toxoplasmosis occurring in Holland in a six-week-old infant, in whom during life toxoplasma could be shown in the cerebrospinal fluid by smear and also isolated by mouse inoculation. The serum of the infant's mother showed a strongly positive neutralization reaction, which was carried out by Sabin's rabbit skin test. In cross-neutralization tests and cross-immunity experiments on rabbits, the Dutch strain appeared to be closely related, perhaps identical with an American one.

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Diffusion Constants of the *E. coli* Bacteriophages.

A. POLSON.* (Introduced by Ralph W. G. Wyckoff.)

From the Laboratory of Physical Biology, National Institute of Health, Bethesda, Md.

For determining the diffusion constants of substances which can be obtained pure in relatively large amounts, the conventional optical method of Lamm is most suitable. Unstable substances like bacteriophages and animal viruses, however, are often irreversibly altered by the purification procedures, and diffusion measurements on such systems

are then subject to large errors. The diffusion of such substances is better investigated using impure solutions that are quantitatively evaluated through measurements of their biological activities.

Hershey, Kimura and Bronfenbrenner¹ have estimated the relative sizes of T¹ and T²

* Permanent address: Onderstepoort Veterinary Research Laboratories, South Africa.

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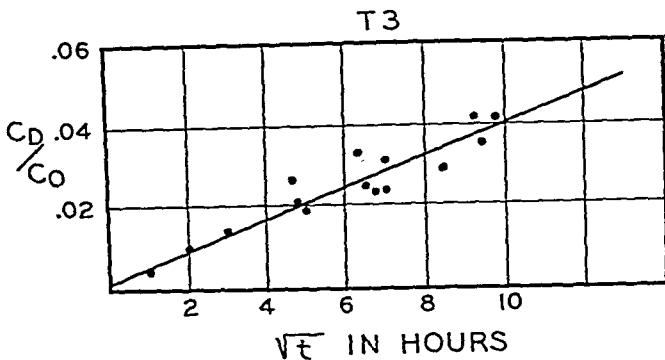


FIG. 1.
Ratio C_D/C_0 plotted against the \sqrt{t} for T_3 bacteriophage.

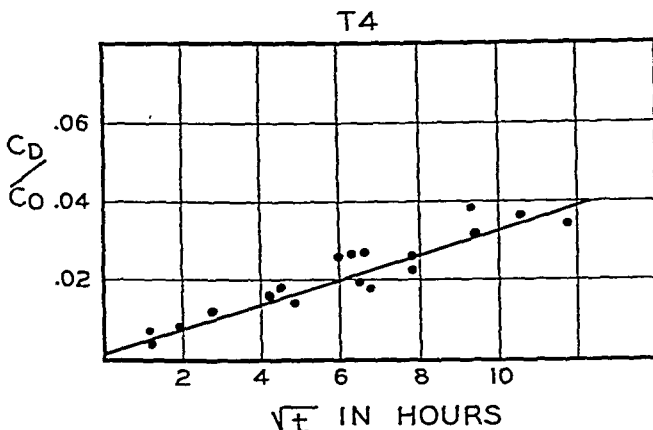


FIG. 2.
Ratio C_D/C_0 plotted against the \sqrt{t} for T_4 bacteriophage.

bacteriophages against *E. coli* by measuring their diffusion through thin agar, but such measurements are difficult to evaluate because of the different adsorption of these bacteriophages by the pores of the agar. The present paper is a preliminary account of the application of another diffusion technique to a tailed, T^4 , and a tailless, T^3 , bacteriophage. These bacteriophages were chosen to find out if the tail influences diffusion as well as to compare particle sizes computed from diffusion measurements with those measured with the electron microscope.

The method is essentially that previously employed by the author to determine the diffusion constant of African horse sickness virus.^{2,3} The apparatus consists of 4 multi-

chambered diffusion cells clamped onto a base plate provided with levelling screws. Sharp interfaces between bacteriophage and medium were formed by filling adjacent cells with medium and bacteriophage suspension and by rotating the top sections of the cells relative to the bottom until the 2 liquids came into exact apposition to one another. This was done by watching the index lines on the sides of the cells. The bacteriophage solution was diluted with 1% glucose broth solution to increase boundary stability and to insure a diffusion process free from disturbances by convection. This glucose broth solution, due to its higher density than the medium above it, forms a sharp boundary in the cell; the high rate of diffusion of its glucose into the broth produces a density gradient in the chamber into which the phage diffuses.

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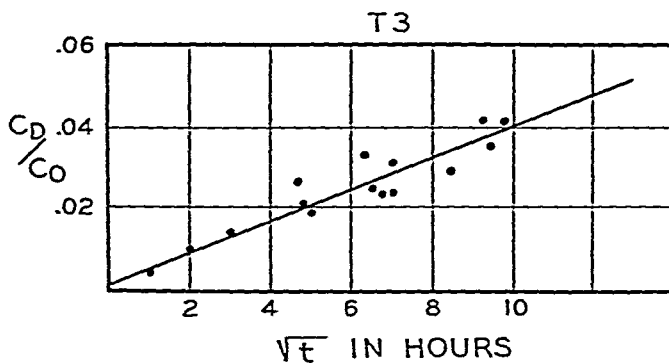


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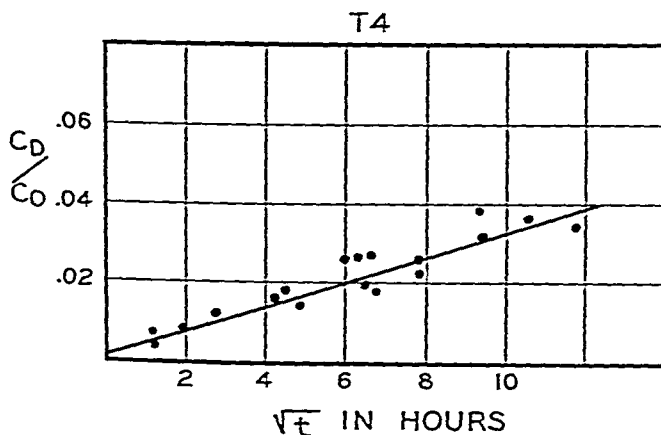


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TABLE I.

Phage	$D \times 10^7 \text{ cm}^2/\text{sec}(\text{obs.})$	η_m	T	$D_{20} \times 10^7 \text{ cm}^2/\text{sec}(\text{corr.})$	Particle diameter in μ
T ₃	1.19	0.0101	295	1.19	36.2
T ₄	0.798	0.0101	295	0.798	55.0*

* This figure is an equivalent spherical diameter, since this phage T₄, due to its tail, is obviously non-spherical.

fused past the initial boundary was isolated from that in the bottom by rotating the top sections to their cut-off position. The phage contents of the diffusates as well as of the original material in the lowest section were then determined by the usual plaque-count method.

The results are given in graphical form in Fig. 1 and 2, where the ratios of concentration in the diffusates C_D to that in the original material C_0 are plotted against the square root of t , the time in hours. It is clear from these figures that C_D/C_0 is a linear function of \sqrt{t} , a necessary requirement from the laws of diffusion in solutions.

Average diffusion constants were calculated from these data using the equation:³

$$D = (C_D/C_0)^2 \cdot \frac{H^2 \pi}{t}$$

where t is the time of diffusion in seconds, and H is the height of the column of liquid above the original boundary, regarding the chambers in the diffusion cells as cylinders. The value of H in these experiments has been 3 cm. Diffusion constants at the temperature of the experiment (22°C) calculated in this way are given in the second column of Table I. Constants for 20°C have been calculated from these with the expression:

$$D_{20} = D_{22} \cdot \frac{293^\circ \text{K}}{295^\circ \text{K}} \cdot \frac{\eta_m}{\eta_w}$$

where D_{20} is the diffusion constant at 20°C, η_m is the viscosity of the medium, and η_w is the viscosity of water at 20°C.

Particle sizes were computed from the well-known Einstein equation for the diffusion of a spherical particle:

$$D = \frac{RT}{N} \cdot \frac{1}{6 \pi r \eta}$$

where R is the gas constant, T is the absolute temperature, N is Avogadro's number, r is the radius of the particle, and η is the viscosity of the medium. This yields the diameters of the last column of the table. They are to be compared with the diameters of T₃, 45 μ , and of T₄, 65 x 80 μ , as measured from previous electron micrographs.⁴

It is clear that the tail of the bacteriophage T₄ does not contribute to its motion in a liquid medium. The slightest propulsion that such a tail might give the particle would have increased its diffusion constant tremendously since diffusion increases as the square of the distance moved in Brownian motion.

⁴ Delbrück, M., *Biol. Rev.*, 1946, **21**, 30.

A Free Swing Writer for Recording With Very Light Pressure on a Smoked Surface.

ALBERT E. AFFORD. (Introduced by S. Goldschmidt.)

From the Department of Physiology, School of Medicine, University of Pennsylvania.

The writing device to be described was developed for use in situations where record-

ing on a smoked surface was unsatisfactory with the conventional type of "fixed" writing

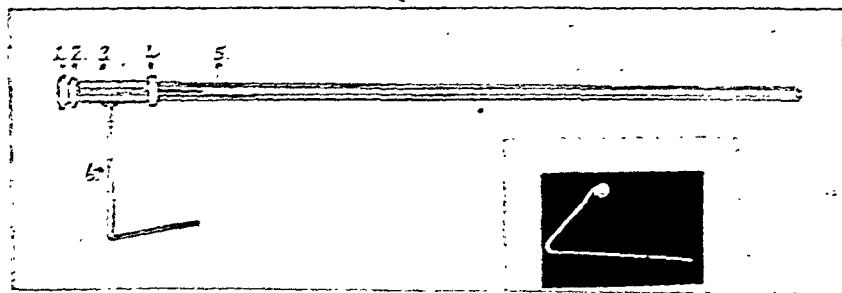


FIG. 1.

The writing device (side view) attached to reed. 1. Pin—length 2 cm. 2. Collar (glass bead), bore 1 mm, O.D. 2 mm. 3. Roller bearing (glass bead), bore 1 mm, O.D. 2 mm, length 6 mm. 4. Collar (glass bead), bore 1 mm, O.D. 2 mm. 5. Straw reed, bore 1 mm, O.D. 2 mm, 5 cm long. 6. Writing point—aluminum wire—gauge 24, 8 cm long. Attached to bearing with sealing wax. Approximate weight 200 mg. The insert shows an end view photograph of the writer.

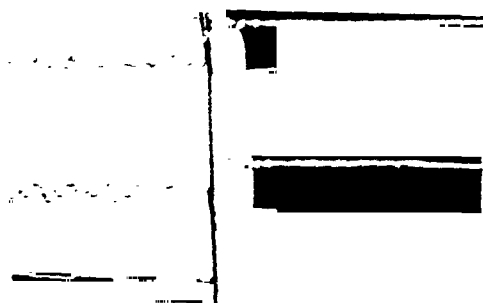


FIG. 2.

Actual tracings and arrangement for recording simultaneously the jugular pulse and the brachial artery utilizing the "free swing" writing lever attached to a tambour (not shown).

point, because of frictional resistance. It has proved to be eminently more satisfactory, in the hands of students, than any device heretofore used. This is especially true in the recording of arterial and jugular pulse tracings (Fig. 2) and esophageal, gastric and duodenal movements in man. It alone has given acceptable tracings of the movements of an excised heart of a clam (*Venus mercenaria*) (Fig. 3). The device (Fig. 1) is exceedingly simple and can be assembled in any laboratory. The writer is a short length (4 cm) of aluminum wire (24 gauge) bent to form an angle of approximately 60° in a plane at right angles to its point of suspension. The angle of the wire forms the writing surface. The pressure of the writer on

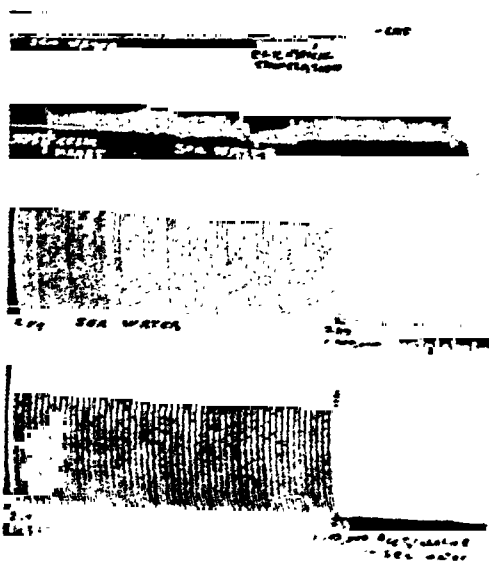


FIG. 3.

Kymograph tracings of excised heart of a clam (*Venus mercenaria*) beating in sea water, utilizing the "free swing" writer attached to light muscle lever.

the kymograph paper may be varied by altering the angle and the length of the free end of the aluminum wire; also by placing small bits of plasticine on the free end of the wire. The writer is attached with deKotinsky cement or sealing wax to a short length (6 mm) of glass tubular bead (1 mm bore), smoothed at the ends, which fits and moves freely over a brass-plated pin about 2 cm

TABLE I.

Phage	$D \times 10^7 \text{ cm}^2/\text{sec}(\text{obs.})$	η_m	T	$D_{20} \times 10^7 \text{ cm}^2/\text{sec}(\text{corr.})$	Particle diameter in $m\mu$
T ₃	1.19	0.0101	295	1.19	36.2
T ₄	0.798	0.0101	295	0.798	55.0*

* This figure is an equivalent spherical diameter, since this phage T₄, due to its tail, is obviously non-spherical.

fused past the initial boundary was isolated from that in the bottom by rotating the top sections to their cut-off position. The phage contents of the diffusates as well as of the original material in the lowest section were then determined by the usual plaque-count method.

The results are given in graphical form in Fig. 1 and 2, where the ratios of concentration in the diffusates C_D to that in the original material C_0 are plotted against the square root of t , the time in hours. It is clear from these figures that C_D/C_0 is a linear function of \sqrt{t} , a necessary requirement from the laws of diffusion in solutions.

Average diffusion constants were calculated from these data using the equation:³

$$D = (C_D/C_0)^2 \cdot \frac{H^2 \pi}{t}$$

where t is the time of diffusion in seconds, and H is the height of the column of liquid above the original boundary, regarding the chambers in the diffusion cells as cylinders. The value of H in these experiments has been 3 cm. Diffusion constants at the temperature of the experiment (22°C) calculated in this way are given in the second column of Table I. Constants for 20°C have been calculated from these with the expression:

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It is clear that the tail of the bacteriophage T₄ does not contribute to its motion in a liquid medium. The slightest propulsion that such a tail might give the particle would have increased its diffusion constant tremendously since diffusion increases as the square of the distance moved in Brownian motion.

⁴ Delbrück, M., *Biol. Rev.*, 1946, **21**, 30.

16281

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ALBERT E. AFFORD. (Introduced by S. Goldschmidt.)

From the Department of Physiology, School of Medicine, University of Pennsylvania.

The writing device to be described was developed for use in situations where recording on a smoked surface was unsatisfactory with the conventional type of "fixed" writing

observed.

The neuro-vasopressor reflex originated by stimulation of the central end of the vagus scarcely altered in the dog pretreated with SY-28.

The diphasic hypertension following electric stimulation of the splanchnic nerve, in normal dogs, is dissociated into a monophasic rise and a secondary fall of blood pressure after injection of 1 mg/kg SY-28. No reversal of the initial neuro-vasopressor response of sympathetic origin occurs.

3. Effects on hypertensive substances

Intravenous injection of 1 mg/kg SY-28 reverses the vasoconstrictor effects of epinephrine and inhibits its tachycardic action.

Same doses reduce or abolish the vasopressor effect of nicotine, ephedrine and veritol (β -p-oxyphenyl-isopropylmethylamine), but never induce a vasodilator response.

The hypertensive action of acetylcholine, in the atropinized dog, persists after injection of SY-28.

The vasopressor action of levo-arterenol[†] is notably depressed, at a time when neuro-vasopressor effects of sympathetic origin are nearly normal and epinephrine reversed by SY-28.

B./ β -(2-biphenyloxyethyl)-n-butyl- β -chloroethylamine HCl (SY-30).

According to Loew's and our observations,

[†] Kindly provided by M. L. Tainter (*Science*, 1948, 107, 39).

the pharmacological actions of SY-30 and SY-28 are similar, the SY-30 being less active.

Intravenous injection of 5 to 10 mg/kg has no immediate effect on blood pressure and heart rate, whereas intra-arterial injection produces a marked local vasodilatation, often preceded by a slight vasoconstriction. Later on the general blood pressure decreases progressively.

Vasopressor effects of carotid sinus origin are rapidly depressed but not reversed. Similarly, the neuro-vasopressor responses due to stimulation of the splanchnic nerve or other sympathetic nerves, may be diminished, but are never reversed after injection of SY-30.

While epinephrine hypertension is reversed and its tachycardic action completely abolished, the vasopressor activity of ephedrine, nicotine, dl-arterenol, acetylcholine (after atropinization) is weakened or blocked.

Finally, SY-30 paralyzes the cardio-accelerator nerves.

Summary. The experiments showed that α -naphthylmethylethyl- β -bromoethylamine (SY-28) and β -(2-biphenyloxyethyl)-n-butyl- β -chloroethylamine (SY-30) are very active adrenolytic agents, with a weak sympathicolytic effect. The present and previous experimental observations once more demonstrate a marked dissociation between the vascular action of epinephrine and the neuro-vascular sympathetic transmission of excitations.

16283 P

Effect of Muscle Work Upon Level of Blood Glucose in the Eviscerated Rat.

DWIGHT J. INGLE.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

These experiments show that faradic stimulation of the gastrocnemius muscle accelerates the rate of fall of blood glucose in eviscerated and eviscerated-nephrectomized rats.

Methods. Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a

weight of 185 to 205 g, the inferior vena cava was ligated between the liver and kidneys to cause the development of a collateral circulation. Asepsis was preserved. When the animals reached 265 to 310 g they were anesthetized (cyclopal sodium) and eviscer-

long. This pin serves as a shaft or axle for the glass bearing to which the writer is cemented. Very smooth glass beads (1 mm bore, 2 mm long) on either end of the glass bearing hold it in place and prevent side motion. The pin is inserted into the end of a reed (1 mm bore, 8 cm long) such as is ordinarily used in kymograph recording. This reed is attached as usual to the recording tambour. Made and suspended as described

above, the writer is free swinging and adapts itself to an uneven surface with uniform and minimal frictional resistance.

Summary. A free swinging writer which records on smoked surfaces with a minimum of frictional resistance is described; its construction is explained and illustrated. Specimen tracings of difficult recordings are illustrated.

16282 P

On New Adrenolytic Compounds.*

G. R. DE VLEESCHHOUWER. (Introduced by C. Heymans.)

From the J. F. Heymans Institute of Pharmacology, University of Ghent, Belgium.

In previous papers¹ we have demonstrated that several so-called sympathicolytic substances have mainly adrenolytic properties. In fact, these substances (benzodioxanes, dibenamine, dihydroergotamine, corynanthine) reverse the epinephrine-hypertension, but do not suppress sympathetic-vasopressor effects.

The present experiments were undertaken with two new synthetic compounds: α -naphthylmethylethyl- β -bromoethylamine HBr (SY-28) and β -(2-biphenyloxyethyl)-*n*-butyl- β -chloroethylamine HCl (SY-30).[†]

The adrenolytic properties of these compounds were demonstrated by Loew, Achenbach and Micetich.^{2,3,4}

Methods. The experiments were performed on chloralosed dogs. The blood pressure was recorded from a femoral artery. All

drugs were injected intravenously and sometimes intra-arterially.

Results. A./ α -naphthylmethylethyl- β -bromoethylamine HBr (SY-28).

1. *Effects on blood pressure, heart rate and respiration.* Intravenous injection of 1 mg/kg SY-28 produces an instant, moderate, transient hypotension and no change of heart-rate. The respiration becomes slow and irregular during the injection, but after a few minutes the dog is breathing normally.

Intra-arterial injection of small quantities of SY-28 (the animal being prepared according to the 3-manometers-method⁵) induces a marked and local vasodilatation.

Some hours after intravenous injection of SY-28, one remarks a slow and progressive fall of blood pressure. This corroborates Loew's observations, but we believe this hypotension to be due to a central depressing action of the drug.

2. *Effects upon neuro-vasomotor reactions.*

The vasopressor and vasodepressor reflexes of carotid sinus origin are slightly depressed after injection of 1 mg/kg SY-28, while vasopressor effects of epinephrine are completely reversed. Although reversal of carotid sinus vasopressor reflexes never appears, a slow progressive depression of these reflexes has

* Aided by grants of the Ella Sachs Plotz Foundation, New York.

† Kindly supplied by Dr. Earl Loew and Parke, Davis & Co.

¹ De Vleeschhouwer, G. R., *C. R. Soc. Biol.*, 1933, **115**, 187; 1934, **115**, 1247; 1935, **118**, 792; *Arch. int. Pharmacodyn. et Théor.*, 1935, **50**, 251; *Proc. Soc. Exp. Biol. and Med.*, 1947, **60**, 151; in press.

² Loew, Earl R., and Micetich, A., *Fed. Proc.*, 1947, **6**, 351.

³ Achenbach, P., and Loew, Earl R., *Fed. Proc.*, 1947, **6**, 304.

⁴ Loew, Earl R., personal communication.

⁵ Nolf, P., *Bull. Acad. Roy. Belg.*, 1902, p. S95.

been observed.

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16283 P

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Methods. Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a

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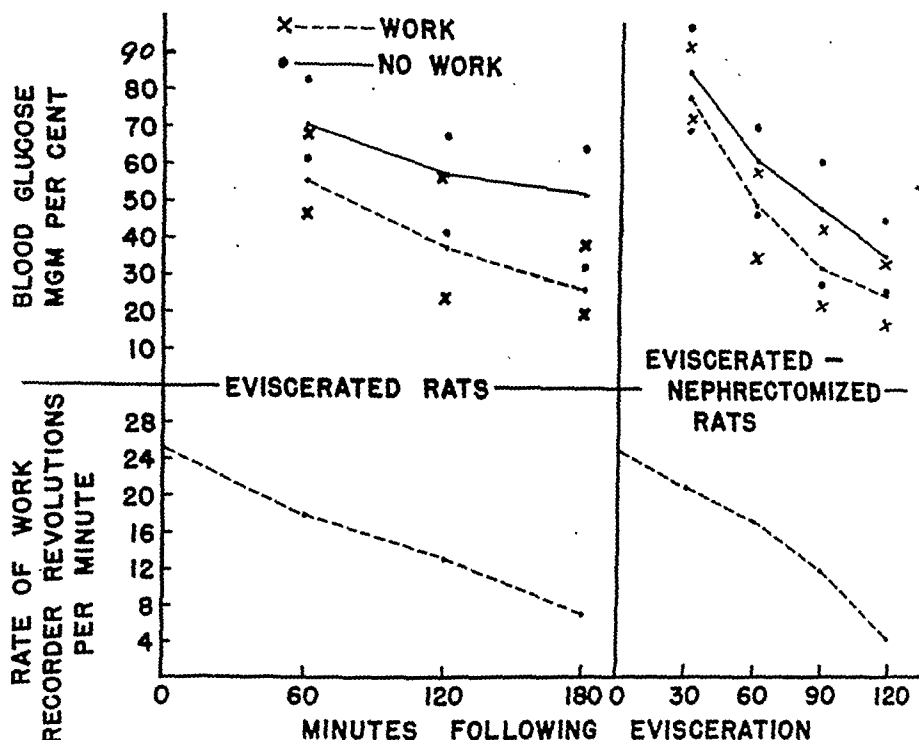


Fig. 1.

The effect of work upon the level of blood glucose in eviscerated and eviscerated-nephrectomized rats. Averages and range. Ten pairs of rats were used in each experiment.

ated by the method of Ingle and Griffith.¹

In Exp. 2 the kidneys were removed at the time of evisceration. Immediately following the operation the animals were prepared for the stimulation of the gastrocnemius muscle to lift 100 g 3 times per second, according to the procedure of Ingle.² The animals were closely matched into pairs on the basis of body weight. One animal of each pair was subjected to work, and the second was kept without the weighting or stimulation of the muscle. Glucose was determined on tail blood by the method of Miller and Van Slyke.³

Experiments and Results. Ten pairs of rats were used in Exp. 1. One rat of each pair was subjected to stimulation of the gas-

trocnemius muscle for 180 minutes. Samples of blood were taken from each animal at the end of 60, 120 and 180 minutes following evisceration.

Ten pairs of eviscerated-nephrectomized rats were used in Exp. 2. One rat of each pair was subjected to the stimulation of muscle for 120 minutes. Samples of blood were taken 30, 60, 90 and 120 minutes following evisceration.

The data are summarized in Fig. 1. The rates of work and levels of blood glucose decreased more rapidly in the eviscerated-nephrectomized rats than in the eviscerated rats. In both series of animals the fall in blood glucose was accelerated by the stimulation of muscle. All of the "work" series developed convulsions and fatal hypoglycemia before convulsions were shown by any of the "no work" series.

Comment. The effect of nephrectomy in hastening the onset of hypoglycemia in the rat was previously known.⁴ Although the

¹ Ingle, D. J., and Griffith, J. Q., Chapter 16. *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

² Ingle, D. J., *Endocrinology*, 1944, **34**, 191.

³ Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

eviscerated rat lacks a pancreas and its hormone, insulin, it is capable of utilizing some glucose, as is readily shown when the organs responsible for the endogenous formation of glucose, *i.e.*, the liver and kidneys, are absent. The rate at which such animal removes glucose from the blood is further accelerated by

⁴ Reinecke, R. M., *Am. J. Physiol.*, 1942, **136**, 167.

muscular work. We propose to study the effect of muscle work upon the glucose load which the eviscerated rat can tolerate in the presence and absence of insulin.

Summary. In eviscerated and in eviscerated-nephrectomized rats stimulation of the gastrocnemius muscle accelerates the fall of blood glucose and shortens the time of survival.

16284 P

Enhancing Effect of Mg^{++} on Clotting Activity of Ca^{++}

FRANK MALTANER.

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Recent studies¹ on the activating effect of metallic salts on the hemolytic function of complement led to the conclusion that free bivalent cations were essential to the process and that the importance of Mg^{++} was more decisive than Ca^{++} or other cations studied.

In previous publications we have reported experiments indicating a close correlation between cephalin and Ca^{++} and their role in the coagulative and complementary activities of blood plasma or serum.²⁻⁴ Moreover, in the clotting process the action of ionized calcium salts has been shown to be highly specific in that salts of even closely related elements, strontium and barium, had relatively little effect.

It seemed of importance therefore to determine whether magnesium, although inactive alone, might enhance the coagulative activity of calcium for plasma or serum.

The technic used in these experiments was similar to that described previously³ except for the use of guinea pig plasma instead of

rabbit plasma. Two preparations were employed: a 0.1% cell-free oxalated plasma for determining the direct coagulating effect of Ca^{++} or other ions and a more strongly oxalated and diluted plasma, the dioxalated plasma of Bordet and Delange⁵ which is more resistant to clotting by direct recalcification and provides a satisfactory reagent for the detection of "thrombin" activity.

Oxalated plasma was obtained by bleeding from the carotid artery over paraffined surfaces into 1% sodium oxalate containing 0.5% NaCl, 1 part to 9 parts of blood, and removal of cells and platelets by centrifugation at 2°-6°C.

Dioxalated plasma was prepared by mixing 1 volume of cell-free oxalated plasma with 4 volumes of 0.2% sodium oxalate containing 0.85% NaCl.

The solutions of $MgCl_2$ and $CaCl_2$ were prepared by diluting molar solutions with 0.85% saline.

The cephalin was a 0.01% solution of phosphatidyl serine in 0.85% saline.

In the experiments of Fig. 1, 0.1 ml amounts of the molar concentrations of $CaCl_2$ or $MgCl_2$ indicated were used, the volume was made up to 0.6 ml with saline and 0.1 ml of

⁵ Bordet, J., and Delange, L., *Ann. de l'Institut Pasteur*, 1912, **26**, 657.

¹ Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

² Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1936, **30**, 417.

³ Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

⁴ Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *Am. J. Physiol.*, 1937, **119**, 80.

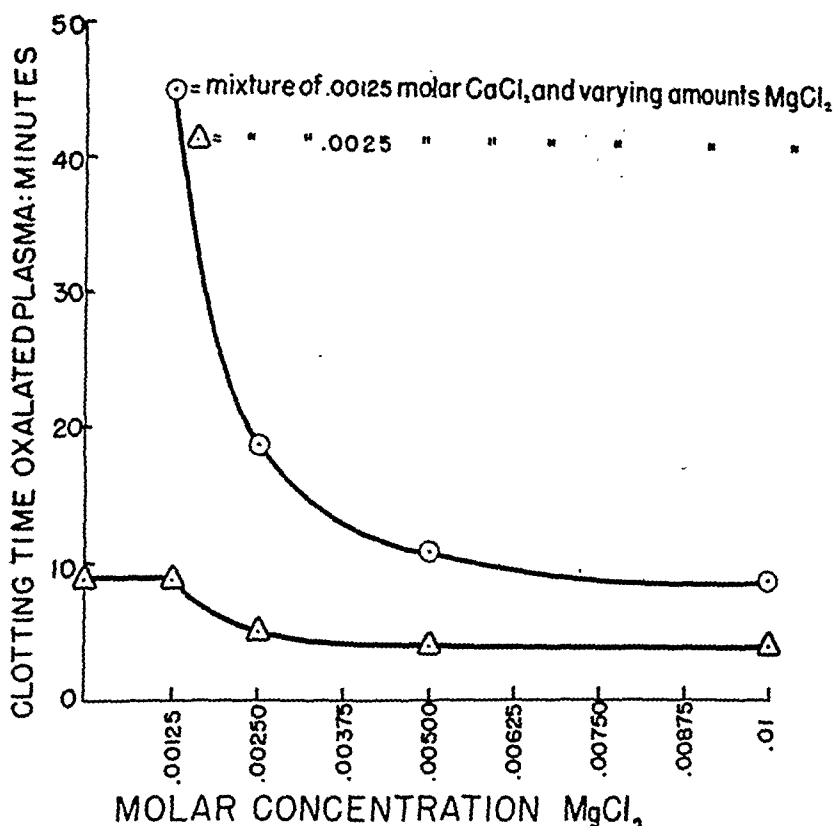


FIG. 1.

The enhancing effect of $MgCl_2$ on the coagulation of recalcified oxalated guinea pig plasma. No clotting was observed with any concentration of $MgCl_2$ without $CaCl_2$ during two hours' observation. 0.00125 molar $CaCl_2$ alone failed to clot within 2 hours.

oxalated plasma was added. Thus the effective molar concentration was one-seventh of that indicated in the figure. The tubes were placed in a $37^\circ C$ water bath and clotting times determined.

In the experiments of Fig. 2 the quantities of guinea pig complement indicated were pipetted from 1:10 and 1:100 dilutions in saline, 0.1 ml of 0.00125 molar $CaCl_2$ and 0.1, 0.2, 0.3, or 0.4 ml of 0.00125 molar $MgCl_2$ were then added, and finally 0.1 ml of a 0.01% solution of phosphatidyl serine. The volume was brought to 0.6 ml with saline. The effective molar concentration of $CaCl_2$ or $MgCl_2$ was thus one-sixth of that indicated in the figure. Mixtures were incubated 6 minutes in a $37^\circ C$ water bath, 0.1 ml of the oxalated and diluted plasma was then added, and the clotting time determined. In

the absence of complement the indicated amounts of other reagents, either alone or in combination, had no clotting action on di-oxalated plasma nor did the complement alone have such activity.

The enhancing effect of $MgCl_2$ on the coagulation of recalcified cell-free oxalated guinea pig plasma is illustrated in Fig. 1.

The effect of $MgCl_2$ on the clotting activity of guinea pig serum (complement) resulting from preliminary incubation of the serum with cephalin and $CaCl_2$, the so-called prothrombin activation, is illustrated in Fig. 2. These results were obtained with minimum quantities of $CaCl_2$. Similar results were obtained when larger doses of $CaCl_2$ were used but the effect of $MgCl_2$ did not appear so great as the optimum dosage of $CaCl_2$ was approached.

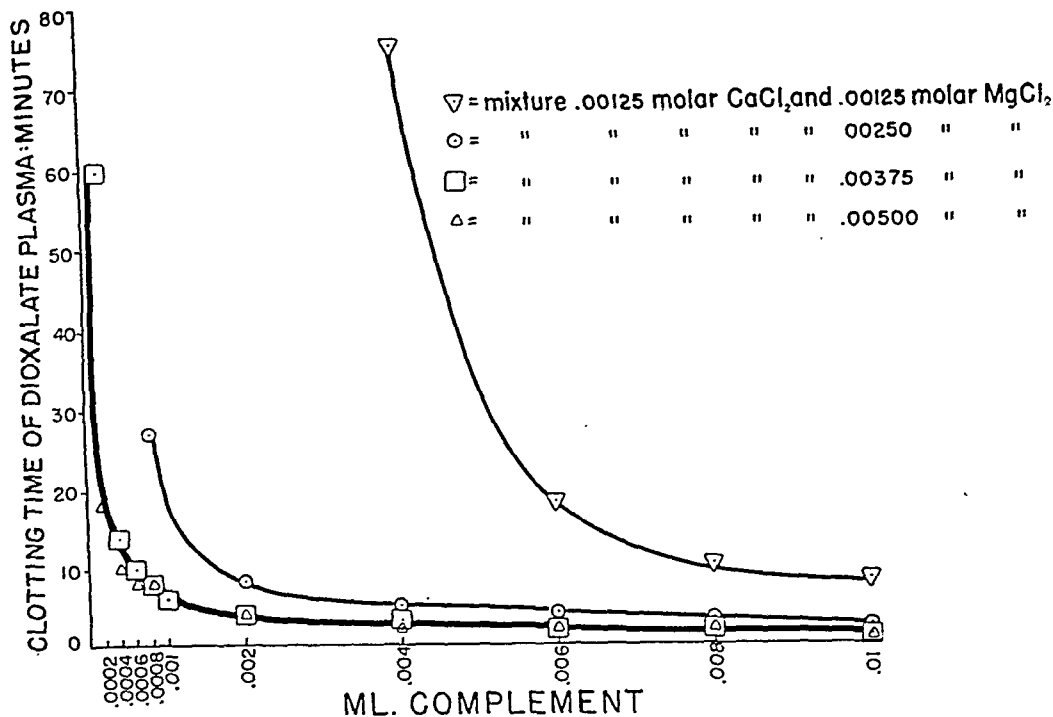


FIG. 2.

Enhancement of clotting activity of serum (complement). No clotting was observed with 0.00500 molar $MgCl_2$ without $CaCl_2$ during 2 hours' observation. 0.00125 molar $CaCl_2$ alone failed to clot within 2 hours.

Conclusions. The results of the experiments demonstrated that whereas ionized magnesium salts ($MgCl_2$) alone do not possess the coagulative activity of calcium salts, they do markedly enhance the effect of calcium. The enhancement is more apparent when the Ca ion concentration is relatively low and the concentration of magnesium salts exceeds that of calcium salts. Thus

the action of Mg^{++} is secondary or accessory, the more essential and decisive role in clotting being played by Ca^{++} .

The parallelism between the enhancing effect of Mg^{++} in the coagulative and the complementary activities of the blood provides additional support for the close relationship of these two phenomena.

Nutritional Requirements of *Trichomonas vaginalis*.*

ALFRED B. KUPFERBERG, GARTH JOHNSON, AND HERBERT SPRINCE.

From the Division of Microbiology, Ortho Research Foundation, Raritan, N.J.

Very little is known about the growth factor requirements of *Trichomonas vaginalis*. In an introduction to the study of growth factor effects Johnson¹ observed that ascorbic acid, glutamic acid and choline had a stimulating effect upon cell multiplication, and that where nicotinamide, folic acid and approximately 10% human serum were employed liver infusion was satisfactorily replaced. Sprince and Kupferberg² reported a medium which could be used as a starting point for the development of a chemically defined culture fluid for the sustained growth of *Trichomonas vaginalis*. A highly complex medium was devised with numerous growth factors included. In these two studies materials of natural origin were added to the media in such concentrations as to mask the essentiality of added *known* growth factors. It is the purpose of the present study to demonstrate that by reducing the serum and trypticase content of the medium of Sprince and Kupferberg a requirement for pantothenic acid and for phosphate was established for *Trichomonas vaginalis*. It was thought that an effort to simplify the above culture medium might reveal a requirement for certain of the component growth factors. Furthermore, the need for a medium to be used for routine maintenance of cultures as well as for testing the antitrichomonas activity of compounds made a cheaper medium desirable. The present study reports the effects of withdrawal of the above growth accessory

substances upon cell multiplication of bacteria-free *Trichomonas vaginalis*.

Methods. Strain No. 2 of bacteria-free *Trichomonas vaginalis* was used as the test organism. The basal medium consisted of the basal trypticase medium previously reported by Sprince and Kupferberg.² The inoculum was prepared by washing cells grown for 48 hours in the trypticase medium. This was accomplished by centrifuging and resuspending in sterile Ringer's solution 4 times. The population was not adjusted. The inoculum consisted of 0.03 ml of the washed suspension. Five tubes were employed for each experimental series except where otherwise indicated. The contents of the tubes were agitated with the tip of the pipette in order to disperse the serum and inoculum. Subcultures were made at 48 hour intervals. Populations were determined by hemocytometer count in the second transplant following the first culture. This medium contained 2 ingredients of natural origin, serum and trypticase. An effort was first made to determine the minimal amounts of serum required for sustained culture in order that sensitivity to withdrawal of the known vitamins might be determined.

Effect of reduction of serum concentration. In order to increase the accuracy of measurement the serum was first diluted to various concentrations in 100 ml of modified Ringer's solution containing 0.6% NaCl and 0.01% NaHCO₃, KCl and CaCl₂. The diluted serum was then added in 2 ml volumes after Seitz filtration. The effects of graded amounts of human serum upon cell multiplication are shown in Table I. Similar results were obtained in a duplicate series. Since the cell counts in cultures containing 0.05 ml of serum were more uniform than those in which the minimal 0.03 ml was used, it was decided to employ 0.05 ml in the experiments to follow.

* The technical assistance of Mrs. Mary Williams, Miss Ruth Grossman, and Mr. LeRoy Markle is hereby acknowledged.

We wish to thank the staff of the Division of Bacteriology and Serology of the State of New Jersey Department of Health, Trenton, for generous amounts of human blood serum.

¹ Johnson, J. G., *J. Parasitol.*, 1947, **33**, 189.

² Sprince, H., and Kupferberg, A. B., *J. Bact.*, 1947, **53**, 435.

TABLE I.
Effect of Graded Amounts of Serum on Cell Multiplication of *Trichomonas vaginalis*.

Ml undiluted serum per 10 ml final medium	Population in second transplant at 48 hr (cells/mm ³)*				
	1	2	3	4	5
.01	0	0	0	0	0
.03	31	57	101	112	88
.05	470	410	240	450	440
.07	1310	1240	700	1190	760
.10	1910	1440	1860	2090	2190
1.0	2510	2470	2310	2270	2310

* Similar results were obtained in a duplicate series.

TABLE II.
Effect of Omission of Vitamins in Groups.

	Population of <i>Trichomonas vaginalis</i> in second transplant at 48 hr (cells/mm ³)*				
	1	2	3	4	5
Control	350	190	280	360	280
" minus Group A	0	0	0	0	0
" " " B	390	460	190	400	400
" " " C	440	450	440	480	400

* Similar results were obtained in a duplicate series.

The effect of withdrawal of known growth factors. These materials were first deleted in groups. Where populations were affected by the withdrawal of a group of compounds, the effect of withdrawal of individual compounds was then explored.

Results. Preliminary studies showed that thiamine HCl, p-aminobenzoic acid, pyridoxine HCl, pyridoxamine HCl, pyridoxal HCl and inositol were not required for sustained growth in the presence of trypticase and serum. The withdrawal of sodium acetate, sodium bicarbonate, asparagine, ribose, adenine sulfate, guanine HCl, xanthine, and uracil was also without effect upon the growth of *Trichomonas vaginalis*. These materials were therefore deleted in the following study of essentiality of vitamins. In the experimental study upon withdrawal of 3 groups of vitamins, however, a specific vitamin requirement was uncovered. The compounds in this study were grouped as follows with the amount of each given in μ g per 10 ml of final medium: A. Choline 80 μ g, riboflavin 8.0 μ g, calcium pantothenate 3.2 μ g, nicotinic acid 3.2 μ g; B. Biotin 0.8 μ g, folic acid 0.8 μ g; C. Ascorbic acid 1000

μ g. The control medium contained groups A, B, and C plus trypticase 2% and serum 0.5%. Agar, maltose, cysteine HCl, and Ringer's were present in the same concentrations as in the original trypticase medium. These groups were then withdrawn from the medium separately. The results obtained are shown in Table II.

It is demonstrated in Table II that one or more vitamins in Group A are essential. Growth equal to that of the control was obtained where Groups B and C were omitted from the medium. The vitamins in Group A, namely: choline chloride, riboflavin, calcium pantothenate and nicotinic acid were then withdrawn singly. The effect of single omissions of these 4 vitamins may be noted in Table III. From these data we may conclude that pantothenic acid is an essential metabolite of *T. vaginalis*. In Table IV it is also shown that the effect of pantothenate is not dependent upon the presence of added vitamins.

It has been demonstrated by Denko *et al.*³

³ Denko, W. D., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, 13, 451.

TABLE III.
Effect of Omission of Single Vitamins.

Vitamins omitted from control	Population of <i>Trichomonas vaginalis</i> in second transplant at 48 hr (cells/mm ³)*				
	Cultures				
	1	2	3	4	5
None (control) †	710	570	540	950	720
Choline	620	620	940	550	660
Riboflavin	630	610	900	660	690
Nicotinic acid	630	880	540	670	870
Ca pantothenate	1	2	0	0	0

* Similar results were obtained in a duplicate series.

† Same as control in Table II except Groups B and C omitted.

TABLE IV.
Effect of Increase in Serum and Omission of All Known Vitamins.

Simplified medium†	Population of <i>Trichomonas vaginalis</i> in second transplant at 48 hr (cells/mm ³)*				
	Cultures				
	1	2	3	4	5
0.5% Serum	0	0	0	0	0
Ca Pant. and 0.5% Serum	780	530	640	620	580
Minus Ca Pant., plus 5% Serum	2150	1950	1890	1920	2040

* Similar results were obtained in a duplicate series.

† Unessential ingredients omitted from medium.

TABLE V.
Effect of Graded Amounts of Trypticase on Cell Multiplication of *Trichomonas vaginalis*.

% Trypticase in final medium	Population in second transplant at 48 hr (cells/mm ³)			
	Cultures			
	1	2	3	4
.008	10	9	7	14
.04	64	70	62	64
.08	151	108	111	165
.16	230	300	350	240
.24	450	420	410	420
.48	940	540	870	810
.72	1210	1300	1020	990
.96	1500	1600	1560	1500
1.20	1830	1640	1760	1620
1.36	1750	1810	1830	1460
1.60	1970	1750	1860	1680
2.00	2050	1920	1970	1750

that normal whole blood has on the average 33 μ g of pantothenic acid per 100 ml. The effect of increased amounts of blood serum upon the requirement for added pantothenate was therefore explored. The medium was prepared minus the addition of all known vitamins and the serum content was increased from 0.5% to 5.0%. The results are indicated in Table IV.

As is shown in Table IV no need exists for Ca pantothenate when 5% human serum is employed in the medium in the presence

of 2% trypticase (BBL).

Effect of reduction of trypticase concentration. Using the basal medium of Sprince and Kupferberg² the trypticase content was varied between 0.008 and 2.0% in the presence of 5% serum. The effect of these reductions is shown in Table V.

As can be seen from Table V, 0.24% of trypticase gave a uniform cell count. In several duplications of this experiment, trypticase concentrations below this value produced widely varying populations in the quadru-

TABLE VI.
Effect of Simultaneous Reduction of Serum and Trypticase Concentrations.

Conc. % final vol. Trypticase	Serum	Population cells per mm ³				
		Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
2	5	1980	2100	2300	1980	1870
2	0.5	680	700	610	690	690
0.24	5	560	440	480	440	470
0.24	0.5	0	0	0	0	0

TABLE VII.
Effect of Addition of Phosphate to Medium Containing Reduced Serum and Trypticase.

	Population cells per mm ³				
	Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
Serum 0.5%— Trypticase 2%	970	760	930	860	880
Serum 0.5%— Trypticase 0.24%	0	0	0	0	0
Serum 0.5%— Trypticase 0.24% KH ₂ PO ₄ 0.027%	276	304	315	260	270

pligate cultures with aberrations in shape and motility. For these reasons 0.24% trypticase was selected for use in further investigations.

Effect of simultaneous reduction of serum and trypticase concentrations. From the foregoing data independent reduction of serum to 0.5% and of trypticase to 0.24% is shown to produce low but consistent populations. The effect of simultaneous reduction of serum and trypticase to these low values is shown in Table VI.

The above data reveal that this medium which contains a full complement of added growth factors is deficient in some respects when serum is reduced to 0.5% and trypticase to 0.24%. It was then supplemented with a variety of mineral salts in order to determine whether this deficiency was due to a lack of an essential salt. Table VII shows the effect of the addition of phosphates.

The data in Table VII demonstrate a requirement for phosphate. Substitution of NH₄H₂PO₄ gave results comparable to those produced by the salts of potassium. The addition of MgSO₄, FeSO₄, MnSO₄ and MnCl₂ was without effect. The optimal concentration of phosphate was found at

0.027%. At this phosphate level the population of trichomonads was approximately 30% of that obtained with 2% trypticase.

Reduction of components in routine culture medium. From the data gathered in the foregoing study it is obvious that in a medium containing adequate serum and trypticase, added growth factors are not required. The trypticase also supplies sufficient phosphate. In view of this fact, the need for the salts included in the Ringer's solution was investigated. Deletion of the Ringer's solution produced no change in the ability of the medium to support optimal populations in serial cultures.

Composition and Population of STS medium. The elimination of all non-essential components left a Simplified Trypticase Serum medium having the following composition per 1000 ml of final medium: Trypticase (BBL) 20 g, cysteine HCl 1.5 g, maltose 1.0 g, Difco agar 1.0 g, distilled water to make 950 ml. The medium was adjusted to pH 6.0, with 1N HCl or 1N NaOH, heated in a boiling water bath until the agar was completely dissolved. The solution was filtered while hot through porous Reeve-Angel filter paper No. 845. To the warm filtered mixture was now added 0.6 ml of

0.5% methylene blue to serve as an indicator. The use of an indicator is optional. After being cooled to 46°C the mixture was re-adjusted, if necessary, to pH 6.0 using the Beckman pH meter. The solution was then brought back to 950 ml with distilled water, tubed in 9.5 ml volumes, autoclaved at 15 lb pressure for 15 minutes, and allowed to cool. To render the medium complete 0.5 ml of sterile, undiluted human serum was added to each tube giving a final volume of 10 ml.

Populations were counted through 11 serial cultures and compared with those in the original medium of Sprince and Kupferberg. It was found that the simplified medium supported populations equal to those in the original medium. Over 50 serial transfers have been maintained to date without visible differences in shape, size and degree of motility of the protozoa. Examination of living specimens by phase microscopy and stain techniques failed to show any difference in

morphology. Three strains of *T. foetus*[†] and one strain of *T. gallinae* have been carried successfully in this medium at this laboratory. The former species requires adjustment of the medium to pH 7. It has recently been observed that all 3 species are satisfactorily supported by Baltimore Biological Laboratory Thioglycollate medium with dextrose and Eh indicator. This medium contained phytone and trypticase. It requires adjustment to pH 6 for *T. vaginalis* and *T. gallinae*. All 3 species require the addition of serum.

Conclusions. 1. Pantothenic acid has been demonstrated to be an essential metabolite for *Trichomonas vaginalis*. 2. A requirement for phosphate has been established. 3. A culture medium containing a greatly reduced number of components has been developed for routine use.

[†] Received from Dr. B. B. Morgan, University of Wisconsin.

16286

Turnover Rate of Phospholipid Phosphorus in the Liver of the White Rat.

JESSE L. BOLLMAN, EUNICE V. FLOCK, AND JOSEPH BERKSON.

From the Division of Experimental Medicine, Mayo Foundation, and the Division of Biometry and Medical Statistics, Mayo Clinic, Rochester, Minn.

The purpose of the experiments reported here is to provide a quantitative estimate of the turnover rate of phospholipid phosphorus in the liver of the white rat under normal conditions of equilibrium, that is, while the actual concentration of the phospholipid phosphorus in the liver is not changing.

The concept of "turnover" is one of dynamic equilibrium in which, during an interval of time dt , a number of phosphorus atoms of mass dm' "enter" the hepatic phospholipid molecules, and the same number "leave," so that the net mass of phospholipid phosphorus remains unchanged. The rates concerned are defined as follows:

$$r = \frac{dm'}{dt} \quad (1)$$

$$R = \frac{r}{m} = \frac{dm'}{mdt} \quad (2)$$

where r is the mass turnover rate; R is the proportional turnover rate; dm' is the mass of phospholipid P which is "turned over" in time dt ; m is the mass of phospholipid phosphorus.

The method used for calculation of the rates is based on the formulation of Zilversmit, Entenman and Fishler.¹ These authors

¹ Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, 1943, 26, 325.

analyzed mathematically the changes of specific activity of a substance which is in equilibrium with a single precursor, the activity of which itself may be changing. Their analysis showed that the proportional turnover rate is equal to the rate of change of specific activity of substance at any instant, divided by the difference of the specific activity of precursor and substance at that instant. If one considers the changes occurring over a finite interval of time, instead of the instantaneous relations, the proportional turnover rate is equal to the change per unit time of activity of substance, divided by the difference of the *mean* specific activity of precursor and substance in the interval. So far as we know, this method has not previously been applied for the calculation of any actual turnover rate.

Changes of radioactivity of hepatic phospholipid have been studied in terms of relative activity, that is the ratio of specific activity of phospholipid phosphorus to that of hepatic or plasma inorganic phosphorus, or in relation to the activity of the phosphate administered.²⁻⁴ These indexes, however, are not measures of the true proportional turnover rate, and will appear as changing values during the course of an experiment, or will vary according to the regimen of administration of radioactive material, even when the turnover rate itself is actually constant.

Hevesy and Hahn,² and Hahn and Tyren⁵ and Hevesy⁶ have suggested calculating the percentage renewal of phosphatides of the liver by comparing the activity of the phosphatide P at the end of the experiment with the average activity of the inorganic P during the experiment, under conditions when the specific activity of the inorganic P of the liver does not change much. They reported the percentage of newly formed phosphatides

in the liver of the rat in 4 hours to be 20% with a strong variation from animal to animal. However, the specific activity of the inorganic phosphorus did in fact change considerably during the course of our experiments. Moreover, even if the hepatic inorganic phosphorus, considered as precursor, remains constant, it is still necessary in accordance with the formulation of Zilversmit and associates to subtract the mean activity of the phospholipid P itself.

The method used for calculating *R* is based on the assumption that the hepatic inorganic phosphate is the immediate and only precursor of phospholipid phosphorus or, more to the point, that its specific activity is not materially different from that of the immediate precursor. Evidence that this condition is fulfilled with sufficient closeness to yield a reliable estimate of *R* is given if the value of *R*, when calculated for successive intervals of the experiment, is reasonably stable and shows no time trend. Still more convincing evidence is provided if the calculated value is essentially the same, even if the shape of the activity time curves themselves is markedly altered by changing the rate of administration of the activated material. As will be seen later, both criteria were used in the experiments here reported, and both confirmed the values obtained.

Experimental methods. Adult male white rats weighing approximately 200 g maintained on the stock commercial diet of "friskies" were fasted for 20 hours prior to the administration of P^{32} , and during the subsequent interval before removal of specimens. The P^{32} , obtained from the Massachusetts Institute of Technology, was administered as the dibasic sodium phosphate. In the first series of experiments reported later each rat received by a single intravenous injection 12 microcuries of P^{32} and not more than 0.02 mg of P^{31} . In the second series the P^{32} was administered by continuous intraperitoneal injection at the rate of 3 microcuries per hour. At specified intervals after the first administration the rats were anesthetized with pentobarbital sodium. Blood was withdrawn by cardiac puncture into a heparinized syringe. The liver was quickly removed and frozen

² Hevesy, G., and Hahn, L., *Kgl. Danske Vidensk. Selskab, Biol. Medd.*, 1940, **15**, 66.

³ Artem, Camillo, Sarzana, Gaetano, and Segrè, Emilio, *Arch. Int. Physiol.*, 1935, **47**, 245.

⁴ Chasikoff, I. L., *Physiol. Rev.*, 1942, **22**, 291.

⁵ Hahn, L., and Tyren, H., *Arkiv f. Kemi, Mineral. och Geol.*, 1946, **21** A, No. 11.

⁶ Hevesy, G., *Arkiv f. Kemi, Mineral. och Geol.*, 1947, **21** A, No. 26.

0.5% methylene blue to serve as an indicator. The use of an indicator is optional. After being cooled to 46°C the mixture was re-adjusted, if necessary, to pH 6.0 using the Beckman pH meter. The solution was then brought back to 950 ml with distilled water, tubed in 9.5 ml volumes, autoclaved at 15 lb pressure for 15 minutes, and allowed to cool. To render the medium complete 0.5 ml of sterile, undiluted human serum was added to each tube giving a final volume of 10 ml.

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TABLE II.

es Obtained with Continuous Intraperitoneal Injection of P ³² at Rate of 3 Microcuries per Hour.*					
Hr after starting administration, <i>t</i>	1	2	3	4	6
Rats	1	3	2	3	2
Body wt, g	202	200	200	199	202
Liver wt, % of body wt	3.1	3.7	3.4	3.7	3.5
Mg P per 100 g liver	6.4	7.3	6.4	7.1	6.4
Plasma inorganic P					
Hepatic " "	29.6	26.7	32.9	26.5	31.1
lipid " "	144	121	122	129	127
Specific activity at time <i>t</i>	1,127	1,302	1,308	1,673	1,710
Plasma inorganic P					
Hepatic " "	534	763	1,170	1,509	1,758
lipid " "	13	33	102	123	273
Relative activity, hepatic lipid:					
inorganic P	0.024	0.043	0.087	0.082	0.155
Mean specific activity to time <i>t</i>	262	470	657	824	1,093
Hepatic inorganic P					
lipid " "	6	15	31	53	105
Diff.	256	455	626	771	988
Change of specific activity, lipid P per hour	13	17	34	31	46
<i>R</i>	0.051	0.037	0.054	0.040	0.046

* The explanation given in the footnote to Table I applies also to Table II.

stein¹¹ and which requires boiling alcohol for extraction, generally had a little less radioactivity than did the phospholipids extractable with alcohol-ether at room temperature.

Results. Two series of experiments were performed. In the first, which employed the larger number of animals, the radioactive material was injected intravenously in a single dose; in the second the material was administered by continuous intraperitoneal injection. The ratio of liver weight to body weight, the water content, total lipid extractable with alcohol-ether at room temperature, and the concentration of inorganic phosphate of the plasma were similar and within normal limits in all the animals. The chief figures of interest are summarized in Table I for the group of animals that received a single injection and in Table II for the group that received a continuous injection. The corresponding activity-time curves for hepatic inorganic phosphorus and hepatic phospholipid phosphorus were obtained by graphic smoothing of the observed specific activities in order to depict the course of the changes in these values and also for the calculation of the mean activities. The curves are shown in Fig. 1 and 2. The mean specific activity, for inorganic phosphorus and phospholipid

phosphorus, which is required for the calculation of *R*, was obtained by evaluating the area under the time curve and relating this to the time interval considered. The area may be calculated by counting squares on the graph paper, by planimeter, or any other appropriate scheme. We have found most convenient and accurate the division of the area into strips and the use of Simpson's rule. The figures pertinent to the calculation of *R* are to be found in rows 12 through 16 of the tables. The rate of change of specific activity of hepatic phospholipid per hour (row 15) is obtained by dividing the specific activity at time *t* (row 10) by the time (row 1). The mean specific activities of hepatic inorganic P and hepatic phospholipid P (rows 12 and 13) were obtained from the area under the time activity curves, as explained previously, and their difference is given in row 14. The value of *R* (row 16), the proportional turnover rate for the period up to *t* is

$$R = \frac{\text{Change in specific activity of phospholipid P per hour}}{\text{Mean specific activity of inorganic P} - \text{mean specific activity of phospholipid P}}$$

These were calculated for the first experiment for 1/2, 1, 2 and 4 hours; the corresponding values were 0.049, 0.052, 0.053 and 0.049 respectively, the last being the rate calculated for the experiment as a whole. The rates calculated for the separate intervals 0 to 1/2,

¹¹ Greenstein, J. P., Nucleoproteins, in Anson, M. L., and Edsall, J. T., *Advances in Protein Chemistry*, New York, Academic Press, Inc., 1944, 1, p. 245.

TABLE I.

Values Obtained After a Single Intravenous Injection of 12 Microcuries of P^{32} and Not More Than 0.02 mg P^{31} .*

		0.5	1	2	4
1	Hr after administration, <i>t</i>	0.5	1	2	4
2	Rats	7	7	10	21
3	Body wt, g	189 ± 4.9	188 ± 4.7	214 ± 6.3	202 ± 3.8
4	Liver wt, % of body wt	3.2 ± 0.2	3.5 ± 0.4	3.8 ± 0.2	3.3 ± 0.1
5	Mg P per 100	6.9 ± 0.4	6.8 ± 0.4	7.5 ± 0.4	7.9 ± 0.2
6	g liver				
7	Plasma inorganic P	31.8 ± 2.1	30.2 ± 1.6	31.1 ± 1.3	33.0 ± 1.1
8	Hepatic lipid	137 ± 3.0	139 ± 4.4	133 ± 5.7	137 ± 1.8
9	Specific activity at	3,876 ± 203	2,015 ± 262	1,174 ± 87	744 ± 33
10	time <i>t</i>	3,063 ± 280	2,578 ± 377	1,837 ± 154	1,102 ± 62
11	Relative activity, hepatic lipid P:				
12	inorganic P	0.02	0.05	0.13	0.31
13	Mean specific activity to	2,618	2,641	2,410	1,932
14	time <i>t</i>	30	67	126	211
15	Change of specific activity, lipid P per hr	2,588	2,574	2,284	1,721
16	R	128	133	122	85
		0.049	0.052	0.053	0.049

* All quantities are means for the number of rats given in row 2; the values after the ± are the standard errors of the means. The mean specific activity to time *t* was obtained as explained in the text.

in a mixture of carbon dioxide ice and alcohol and then was pulverized between chilled steel blocks.

An aliquot of the liver was extracted with trichloroacetic acid, with the usual precautions, and the inorganic phosphate was precipitated as the magnesium ammonium salt. Traces of some organic phosphates are included in this precipitate but the amount and the radioactivity of these do not materially affect the value obtained for either the amount or the specific activity. The phosphorus content was determined by the method of Fiske and Subbarow⁷ and the radioactivity was measured with a Geiger-Muller counter of the immersion type.⁸ Specific activity is reported as counts per second per mg P after correction for decay from the time of injection to the time of counting. Direct measurements of the inorganic phosphate in a trichloroacetic acid extract of the plasma were made without the preliminary precipitation.

Approximately 3 g of pulverized frozen liver were ground immediately with sufficient

anhydrous Na_2SO_4 to remove the water.^{9,10} The tissue was then extracted overnight at room temperature with 50 ml of alcohol-ether. The extract was filtered, the residue was washed once with alcohol-ether and twice with ether and the volume was made up to 100 ml. This procedure in the presence of plasma or tissue protein completely separates lipid P from inorganic P. Samples of blood or tissue taken a few minutes after injection of inorganic P^{32} show no radioactivity in the lipid fraction when the activity of the inorganic P is highest. The P^{32} was measured in a suitably diluted aliquot; the P^{31} was determined in duplicate aliquots after preliminary ashing with H_2SO_4 and H_2O_2 . A large aliquot was evaporated to dryness by aeration at room temperature in the presence of a trace of hydroquinone for gravimetric determination of total lipid. Since at least 90% of the P in the dried lipids was found to be chloroform-soluble and since inorganic phosphate was not present, the alcohol-ether extracts were considered satisfactory for these studies. It must, however, be stated that, if the dried liver residue was extracted with boiling alcohol in the Bailey-Walker extraction apparatus for a 3 hour period, 20 mg more of chloroform-soluble P per 100 g of wet liver were obtained. This fraction, which probably includes the phospholipid attached to nucleoprotein described by Green-

⁷ Fiske, C. H. and Subbarow, Yellapragada, *J. Biol. Chem.*, 1925, **66**, 375.

⁸ Wang, J. C., Marvin, J. F., and Stenstrom, K. W., *Rev. Scient. Instruments*, 1942, **13**, 81.

⁹ Fairbairn, Donald, *J. Biol. Chem.*, 1945, **157**, 645.

¹⁰ Channon, H. J., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, 1937, **31**, 1736.

TABLE II.

 Values Obtained with Continuous Intraperitoneal Injection of P³² at Rate of 3 Microcuries per Hour.*

1	Hr after starting administration, <i>t</i>	1	2	3	4	6
2	Rats	1	3	2	3	2
3	Body wt, g	202	200	209	199	202
4	Liver wt, % of body wt	3.1	3.7	3.4	3.7	3.5
5	Mg P per 100	6.4	7.3	6.4	7.1	6.4
6	g liver	29.6	26.7	32.9	26.5	31.1
7	Plasma inorganic P	144	121	122	129	127
8	Hepatic " "	1,127	1,302	1,308	1,673	1,710
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phosphorus, which is required for the calculation of *R*, was obtained by evaluating the area under the time curve and relating this to the time interval considered. The area may be calculated by counting squares on the graph paper, by planimeter, or any other appropriate scheme. We have found most convenient and accurate the division of the area into strips and the use of Simpson's rule. The figures pertinent to the calculation of *R* are to be found in rows 12 through 16 of the tables. The rate of change of specific activity of hepatic phospholipid per hour (row 15) is obtained by dividing the specific activity at time *t* (row 10) by the time (row 1). The mean specific activities of hepatic inorganic P and hepatic phospholipid P (rows 12 and 13) were obtained from the area under the time activity curves, as explained previously, and their difference is given in row 14. The value of *R* (row 16), the proportional turnover rate for the period up to *t* is

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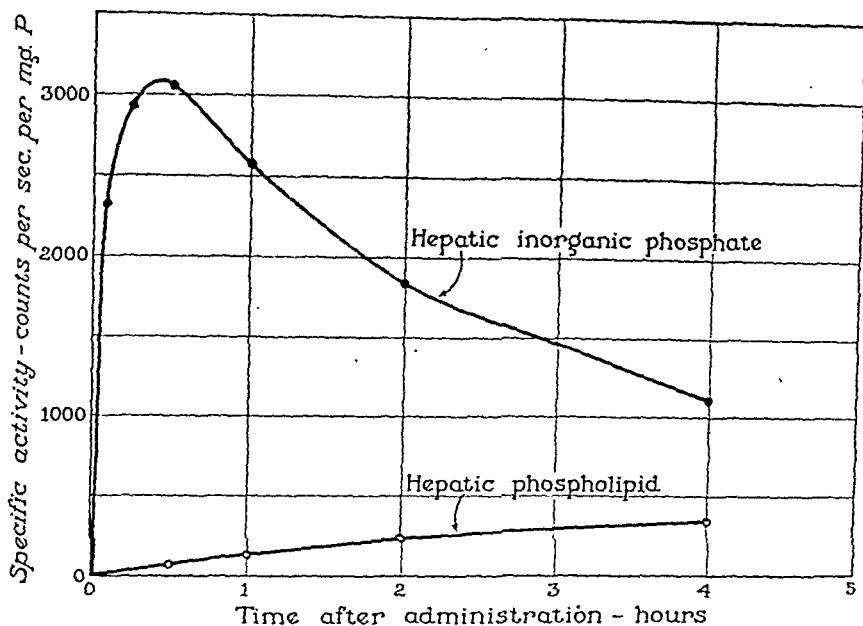


FIG. 1.

Curve of mean values of specific activities found after a single intravenous injection of phosphate P^{32} .

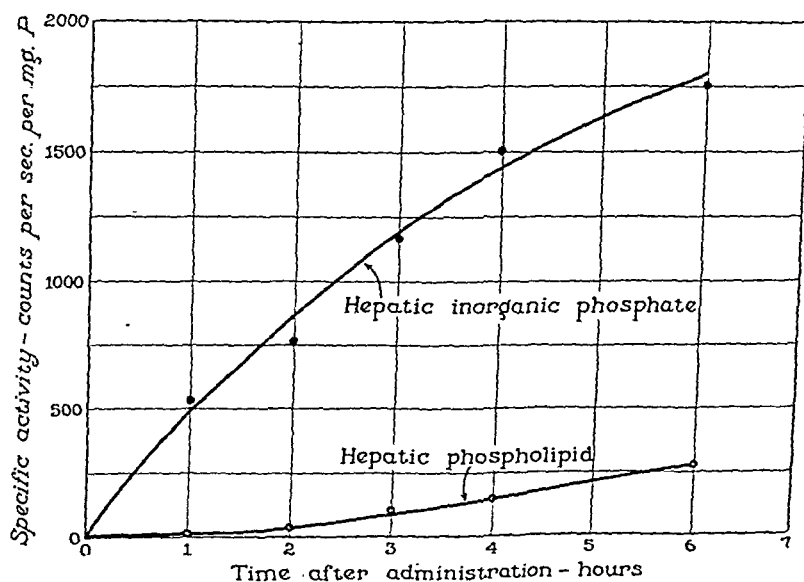


FIG. 2.

Curve of mean values of specific activities found with a continuous intraperitoneal injection of P^{32} .

$\frac{1}{2}$ to 1, 1 to 2, 2 to 4 hours were 0.049, 0.052, 0.053, 0.045. It is seen that this experiment yielded very consistent estimates for R in each of the periods. This is in contrast to

the relative activity (ratio of specific activity of phospholipid P to that of inorganic P) which rose steadily in this period.

The results for the second series of experi-

ments, in which the injection of the radioactive material was continuous, are shown in Table II and the time-activity curves are shown in Fig. 2. It is to be noted that the curves are markedly different from those of the series with the single injection. This result is to be expected. The calculated values of R are given as for the periods up to 1, 2, 3, 4 and 6 hours; these were respectively 0.051, 0.037, 0.054, 0.040 and 0.046, the last representing the result for the experiment as a whole. The figures are not quite so consistent as those for the first series but the variation is not larger than is to be expected, considering the smaller number of animals

used in this series. The relative activity shows no consistency with the first experiment. The figure for R provided by the entire experiment, 0.046, agrees very well with that obtained in the first experiment, 0.049.

Conclusions. The proportional turnover rate R for hepatic phospholipid P in the white rat is on the average close to 5% per hour, and since the mean concentration of hepatic phospholipid P for both experiments together was 132 mg per 100 g of liver, that r , the mass turnover rate, is about 6 mg P per hour per 100 g of liver, which is 0.2 mg P per 100 g body weight.

16287

Growth of Normal Male and Female Mice and of Female Mice Bearing Ovarian Grafts.*†

R. T. HILL. (Introduced by H. G. Day.)

From the Department of Anatomy, Indiana University School of Medicine, Bloomington, Ind.

It has been clearly shown that mouse ovaries, under certain conditions, can supply androgens of sufficient quantity and quality to maintain, in normal conditions, the accessory sex glands of castrate male mice.^{1,2} It has been proven that environmental temperature is a factor in causing grafted ovaries to elaborate androgens, but the exact androgens secreted have never been determined.

It was suggested to the author by Doctor Carl G. Hartman that perhaps female mice bearing active androgenic ovarian grafts might assume a growth pattern comparable to that of normal males. Following this sug-

gestion a group of our animals in experiment were weighed twice weekly, and the weights obtained are presented in Fig. 1. Five normal males and 10 normal females were used as control animals. Twenty-eight females bearing autografts of ovaries in their ears comprised the experimental group. All of the animals were of the same age when started on the experiment. It will be noted that the normal males averaged 16.6 g at the outset of the experiment, while the normal and experimental females averaged 14.9 g. The weights were recorded and plotted for approximately 250 days. At the end of this period the plateau of growth was essentially complete.

A difference of slightly less than 2 g at the outset of the experiment, between males and females, had increased to approximately 4.5 g 60 days later. The difference of 4-4.5 g in the weights of the 2 groups was maintained from then till the termination of the experiment. At no time did the curve obtained

* This work has been supported in part by grants from the VioBin Corporation of Monticello, Ill.; the Graduate School and the Medical Center of Indiana University; and the U. S. Public Health Service.

† The mice used in these experiments were the CHI inbred strain obtained from Dr. L. C. Strong.

¹ Hill, R. T., *Endocrinology*, 1937, **21**, 633.

² Hill, R. T., and Strong, M. T., *Endocrinology*, 1938, **22**, 663.

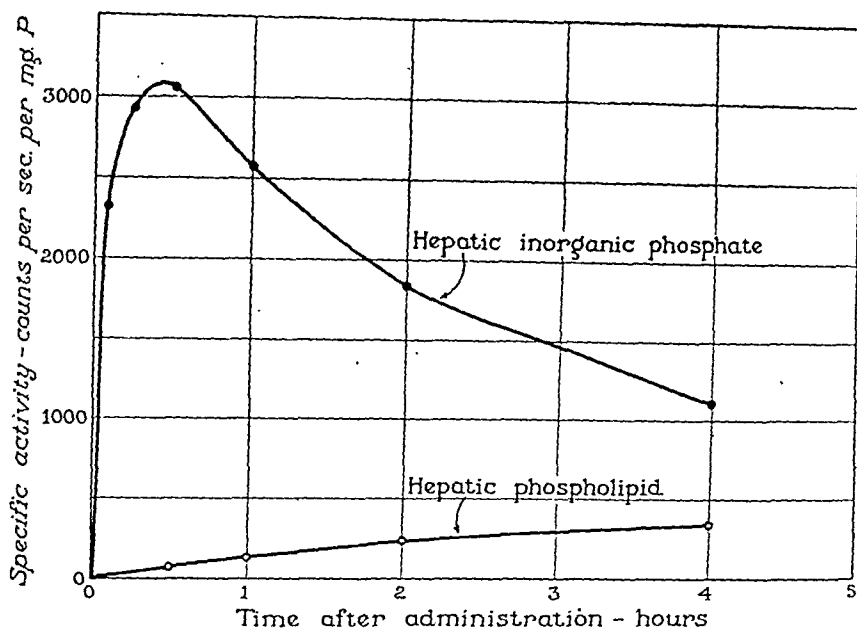


FIG. 1.

Curve of mean values of specific activities found after a single intravenous injection of phosphate P^{32} .

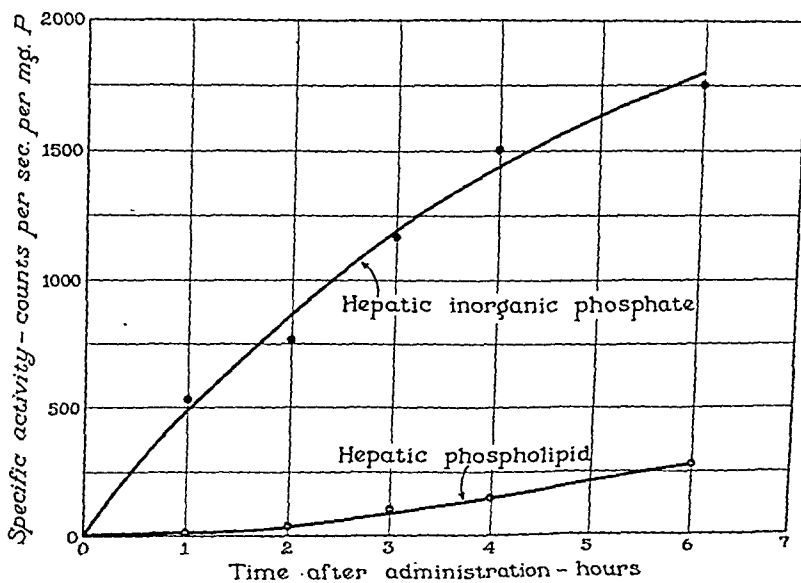


FIG. 2.

Curve of mean values of specific activities found with a continuous intraperitoneal injection of P^{32} .

$\frac{1}{2}$ to 1, 1 to 2, 2 to 4 hours were 0.049, 0.052, 0.053, 0.045. It is seen that this experiment yielded very consistent estimates for R in each of the periods. This is in contrast to

the relative activity (ratio of specific activity of phospholipid P to that of inorganic P) which rose steadily in this period.

The results for the second series of experi-

Storage of Carmine in Mice of Inbred Strains.

K. STERN.

From the Mount Sinai Medical Research Foundation, Chicago, Ill.

Numerous observations have been reported which suggest a relationship between reticulo-endothelial tissues and the development and growth of malignant tumors (lit. cf.).¹ However, discrepant findings and controversial interpretations have as yet not permitted the formulation of a definite concept.

In the present study the attempt was made to approach this problem by making use of the great variation in spontaneous tumor development exhibited by certain inbred mouse strains. For this purpose, animals of the C3H strain with high mammary cancer incidence and animals of the low-cancer strain C57B were employed. The reticulo-endothelial activity of these animals was assayed on the basis of their ability to store lithium carmine.

Carmine (Merck & Co.) was dissolved in a saturated aqueous solution of lithium carbonate to the desired concentration by boiling gently for 3 minutes, followed by filtration through glass wool. Two, 3 and 4% solutions were employed. They were prepared within the 24 hours prior to use and sterilized in the autoclave for 20 minutes (pressure 10-15 lb). The carmine solutions were injected intraperitoneally with concentration and dosage varying in the 5 experimental groups, as shown in Table I. In the first 3 experiments the animals were sacrificed 48 hours after injection, in the last 2 after 24 hours. Sections of liver and spleen were fixed in 10% formalin; in some groups also skin, kidneys and lungs were preserved. Sections of 6 to 8 micron thickness were cut from the formalin-fixed and paraffin-blocked organs. Of each block, a hematoxylin-eosin stained section and a cleared unstained section were prepared. For general observa-

tions, the stained sections were studied. For detailed study of the carmine storage the unstained sections were found best suited.

Particular attention was given to the dye granules taken up by the Kupffer cells in the liver. The over-all impression was that of a more marked storage in Kupffer cells of C57B animals as compared with the findings in C3H mice. In order to express these relations in a roughly quantitative manner, an estimation of the amount of storage was carried out by counting the storing Kupffer cells (SKC) in at least 10 high-power fields and by determining the average of dye-laden cells in each animal. As seen in Table I, this crude quantitative estimation confirmed the impression obtained from general study of the slides.

In all of the 5 experiments, the average numbers of SKC observed in C57B mice exceeded those found in C3H mice. In view of the rather small number of animals making up the single experiments, it is, however, also necessary to analyze the values of SKC in the individual animals. Such an analysis shows that in Experiment I 4 out of 5 C57B mice presented markedly higher SKC values than the 4 C3H mice examined under the same conditions. In Experiment II, 4 out of 6 C57B animals surpassed 6 C3H mice in storing ability. In Experiment III, 1 of the 2 C57B mice showed considerable increase, the other slight increase of SKC as compared with 6 C3H mice. In Experiment IV, the SKC values of 5 out of 6 C57B animals were greater than those obtained in 6 simultaneously tested C3H animals (2 of the latter died 5 hours after the injection of the dye, and hence the small extent of storage probably was due to insufficient time elapsed). In Experiment V, 6 of 7 C57B mice presented higher SKC values than were found in 11 C3H animals under the same conditions. In

¹ Stern, K., and Willheim, R., *The Biochemistry of Malignant Tumors*, Brooklyn, Reference Press, 1943, pp. 696-745.

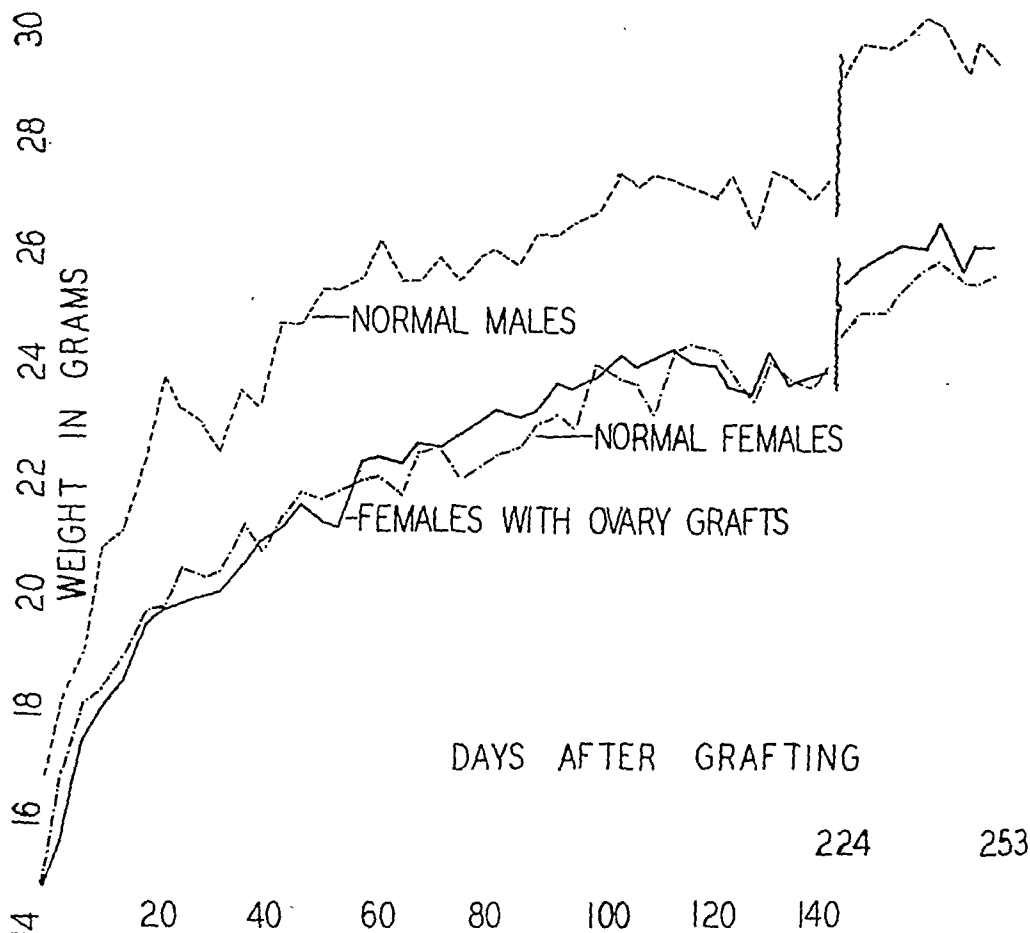


FIG. 1.
Curves representing growth of normal male and female mice and of female mice bearing ovarian grafts.

from the weights of the normal females and that obtained from the grafted females show any significant difference. Likewise, there was no time during the experiment when the growth of the females bearing ovarian grafts

even approached the growth of the normal males.

Summary. Grafts of ovaries in female mice which are androgenically active do not affect the growth weights of the host animal.

relation to the findings just described. The age of the animals ranged from 7 weeks to 10 months; for each experimental group animals of similar age were selected from both strains. The sex distribution was as follows: 17 males and 9 females among the C57B animals; 14 males and 19 females among the C3H animals.

In addition to the liver, the carmine storage was studied in the spleens of all animals. Here, too, a different behavior of the two strains, though less constant and striking, was noted: among C57B animals storage was absent in 2, slight to moderate in 17, and marked in 7; among C3H animals storage was absent in 12, slight to moderate in 21, and marked in none. The storage in the spleen was found mainly in the perifollicular reticulum cells and, to a lesser extent, in littoral cells of the sinuses. Thus halo-like formations around the follicles, consisting of dye-laden cells, resulted from the storage, a distribution which did not lend itself readily to quantitative evaluation. The dye storage in the renal epithelium and the dye excretion within the tubules conformed to the classic descriptions² and no difference between the two strains was detected. Likewise no appreciable difference was observed regarding dye deposits in skin and lungs, which were examined in some groups of animals.

The fact that in these experiments the liver, *i.e.*, the storage in Kupffer cells, presented the most marked difference between the two strains may be due to the route of administration employed, namely, the intraperitoneal injection of carmine. In this procedure, most of the dye is probably absorbed by serosal lymphatics and thus, by way of the portal vein, reaches the liver first. Intravenous injection, which was not used in this study, may possibly lead to different findings.

In previous work at the State Institute for the Study of Malignant Diseases, Buffalo,

N.Y.,* similar experiments were carried out by comparing the carmine storage in C57B mice and in animals of the Marsh-Albino strain. Six animals of each strain were examined. They presented a marked difference in their dye storage in Kupffer cells and in the spleen: heavy, coarsely granular storage was obtained in 5 of the 6 C57B mice and only in 1 of the 6 MA mice.

The C3H and the MA strains are both known for the high incidence of spontaneous mammary carcinoma in the females. On the other hand, this type of tumor is exceedingly rare in the C57B strain. In the latter, the total incidence of all epithelial and non-epithelial spontaneous tumors is around 20% according to studies and records of the Jackson Memorial Laboratory.³ At this time it would be premature to speculate on a possible relationship between susceptibility or resistance to spontaneous cancer development, on the one hand, and the reticulo-endothelial activity of the strain, as expressed in dye storage, on the other. In order to establish such a relationship it will be necessary to extend this work to a number of additional inbred strains with varying rates of spontaneous tumor development.

Summary. The storage of intraperitoneally injected carmine in Kupffer cells of the liver and in the spleens was examined in mice belonging to two inbred strains. Quantitative and qualitative differences were observed which indicated an inferior dye-storing ability of C3H animals as compared with C57B mice.

* I wish here to thank Dr. A. A. Thibaudau and Mr. E. Burke, Department of Pathology, and Dr. S. G. Warner, Mr. M. A. Reinhard, and Miss H. Goltz for their cooperation in providing material and facilities for the work done in Buffalo.

³ Staff of the Jackson Memorial Laboratory, *Biology of the Laboratory Mouse*, The Blunkston Company, Philadelphia, 1941, p. 266.

STORAGE OF CARMINE IN MICE OF INBRED STRAINS

TABLE I.

Exp. No.	Carmine conc. and dosage	Time interval in hrs.	C3H			C57B		
			No. of animals	SKC*		No. of animals	SKC*	
				Values	Mean		Values	Mean
I	3% 0.5 cc (15 mg)	48	4	31		5	54	
				38			67	
				41	38		67	69
				43			76	
							82	
II	2% 0.25 cc (5 mg)	48	6	16		6	22	
				22			35	
				22	25		50	50
				23			60	
				32			62	
				32			71	
III	2% 0.5 cc (10 mg)	48	6	13		2	27†	
				15			41	
				20	19			34
				20				
				21				
				22				
IV	2% 0.5 cc (10 mg)	24	6	5‡		6	24	
				5‡			53	
				10	13		60	69
				10			73	
				19			92	
				26			109	
V	4% 0.5 cc (20 mg)	24	11	10		7	31	
				15			51	
				17			57	
				22			62	
				28			63	
				33	29		64	56
				37			67	
				37				
				38				
				39				
				39				

* Storing Kupffer cells.

† Animal died after 30 hr.

‡ Animal died after 5 hr.

all, 20 out of the 26 examined C57B mice surpassed in carmine storing activity the 33 simultaneously tested C3H mice.

In addition to these quantitative findings, a qualitative difference was frequently noted: finely granular and pale red carmine deposits prevailed in 28 of the 33 C3H mice, with only 5 of them showing dark red, coarsely granular storage, while among the 26 C57B mice 14 presented dark red, coarse granules and 12 exhibited pale red, fine granules. In general, particle size and color intensity tended to increase with the concentration of dye used, though Table I shows that no exact

parallelism existed between dye dosage and SKC values. In livers of C3H mice frequently diffuse distribution of dye in sinusoids was noted. Staining of parenchymatous liver cells (diffuse cytoplasmic stain and darker nuclear stain) occurred in both strains only in isolated areas of necrosis encountered infrequently. The intravital carmine staining of liver cells reported by Kiyono² was accomplished only by repeated dye injections.

No influence of age or sex was apparent in

² Kiyono, K., *Die vitale Karminspeicherung*, Jena, Gustav Fischer, 1914.

TABLE I.
Mitotic Activity in the Hypophysis of Rats.

Group	No. of animals	Age mean	Sex	Inj. of androgen and/or estrogen μg	Mitoses per mm^2 Mean \pm S.E.
1	5	117	♀		1.20 \pm 0.25
2	4	102	♀		1.17 \pm 1.07
3	6	101	♀	3x500A + 2x25E	17.0 \pm 2.76
				3x1000A + 2x25E	
4	10	97	♀		20.0 \pm 1.94
5	10	91	♀		21.3 \pm 1.55
6	6	94	♂	4x500A + 4x17E	18.7 \pm 4.20
7	6	93	♂		20.8 \pm 2.00
8	9	92	♂		.52 \pm .13

females does not change the mitotic activity in the hypophysis (Group 2). The glands of only 4 animals were examined but since in all of these the activity is uniformly low, it seemed unnecessary to extend the series.

In the males and females which received both androgen and estrogen (Groups 3 and 6) the average mitotic activity does not differ significantly from either the male or female groups which received estrogen alone. In comparing Group 3 with 4 statistically, $P = .35$, and 6 with 7, $P = .65$. The animals in Group 3 that had injections of 500 μg of androgen show a somewhat lower activity

than those which had 1000 μg , but the difference is not believed to be important.

Summary. The estrogenic hormone, alpha-estradiol benzoate, stimulates mitotic division in the cells of the anterior hypophysis of both male and female rats and, provided the animals are about the same age, an equal amount injected results in approximately the same mitotic activity. When the androgen, testosterone propionate, is injected with the estrogen in either male or female rats, the mitotic activity is not significantly different from that resulting from injections of estrogen alone.

16290 P

Increased Susceptibility of Mice to Swine Influenza as a Result of Methionine Injections.*

DOUGLAS H. SPRUNT.

From the University of Tennessee College of Medicine, Memphis.

In recent years numerous experiments have been reported on the effects of various dietary constituents on the susceptibility of animals to viral infections. I reported in 1942 that methionine decreased the susceptibility of the rabbit to vaccinia.¹ It seemed advisable however to repeat these experiments in the mouse using swine influenza as the virus. When

mice on an adequate diet are injected with methionine there is a slight increase rather than decrease in susceptibility to swine influenza. But, if methionine were given to mice on a low protein diet, a marked increase in susceptibility occurs. This paper is to report such experiments.

Since poor or malnutrition from many causes will result in an increase in the animal's resistance to viral infections² it was thought

* Aided in part by grants-in-aid from The John and Mary R. Markle Foundation and the United States Public Health Service.

¹ Sprunt, D. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 226.

² Sprunt, D. H., *J. Exp. Med.*, 1942, 75, 297; Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. E.*, 1944, 80, 257.

Mitotic Activity in Male and Female Rat Hypophyses After Combined Injections of Androgen and Estrogen.*

THOMAS E. HUNT.†

From the Department of Anatomy, Medical College of Alabama, Birmingham, Alabama.

Since mitotic activity in the hypophysis of female rats is increased significantly after injections of the female hormone estrogen,¹ the question arises whether the male hormone might not likewise affect the proliferative activity in the hypophysis of either male or female animals. There is some evidence that androgen inhibits the activity instead of stimulating it. Wolfe and Hamilton² found that the pituitaries of rats were only slightly increased in weight after 10 daily injections of both testosterone-acetate and estrone whereas the increase was considerable after the same number of injections of estrone alone.

In the present experiments combined injections of androgen and estrogen were likewise made into rats to see if there is a modification in mitotic activity of the anterior hypophysis. Also the mitotic activity of male rats is compared with that of female rats after equivalent injections of estrogen.

In determining the mitotic activity, all dividing cells are counted in one or more 3 micra coronal sections of the hypophysis and the number present per square mm of section is calculated. The androgen and estrogen used is testosterone propionate and alpha-estradiol benzoate, respectively, both of which were dissolved in sesame oil.† All injections were made subcutaneously. The female animals were ovariectomized approximately one month previous to the time of injections.

* This study was aided by a grant from the Cancer Research Division of the Donner Foundation.

† With the technical assistance of Miss Cecile Morgan.

1 Hunt, T. E., *Anat. Rec.*, 1947, **97**, 127.

2 Wolfe, J. M., and Hamilton, J. B., *Endocrinology*, 1937, **21**, 603.

‡ The estrogen (Progynon-B) and androgen (Oreton) were kindly furnished by the Schering Corporation, Bloomfield, N.J.

The tabulation of the experiments shows the number of animals, the average age of the animals in each group, the hormones injected and the mean and standard error of the mean of the mitoses per sq mm of section. Injections of estrogen were made either 48 and 72 hours before killing the animals or, as in Groups 5, 6, and 7, they were made 28, 36, 52, and 60 hours before killing. In Groups 2 and 3 the androgen was injected 24, 48, and 72 hours before killing, and in Group 6 it was injected at the same times as the estrogen.

The number of dividing cells in the hypophysis of ovariectomized females and in that of normal males is uniformly low in animals 3 months of age or older (Groups 1 and 8). Only 2 or 3 mitotic figures are usually seen and at the most 5 to 10 occur in an entire section that includes 5 or 6 square mm.

Injections of estrogen result in a considerably increased mitotic activity in both ovariectomized females and normal males (Groups 4, 5, and 7). In all 3 groups the average number of mitoses is about 20 per sq mm. Statistically there is no significant difference in the mitotic activity of the male Group 7 and female Group 5, both of which received estrogen ($P = .8$).[§] In the males there is no evidence that the testicular androgen modifies the activity of the injected estrogen. The male hypophysis thus appears to respond to the estrogen about the same as that of the female. However, in the male there is a higher percentage of chromophil cells undergoing division than there is in the female, although, as in the female, the majority of divisions occur in the chromophobes.

Injections of androgen into ovariectomized

§ Fischer's table of P was used to determine statistical significance. P must have a value of less than .05 before a difference can be considered as possibly significant.

TABLE I.
Amount of Weight Lost by Mice on the Various Diets.

Groups	A	B	C	D	E	F
Avg wt in g						
At start of experiment	24.4	24.6	24.0	23.9	23.0	25.4
At time inoculated with virus	21.7	22.1	21.7	21.4	24.2	25.2
At time of death	15.9	—	16.6	—	16.8	—
Surviving mice	20.3	22.6	19.1	21.0	25.1	26.7

essential that the virus dilution be kept at this 50% point in order to assure a number of survivors. Groups B and D remained under similar condition but received no virus. The 4 groups did not differ in food intake and weight. Rockland Mouse Diet which is adequate and has a protein level of 24% was fed the mice in Groups E and F. Inoculation of the mice in Group E was done at the same time and in the same manner as in Groups A and C. The controls, the mice in Group F, received no virus.

The results of the experiment are given in Chart 1. Groups B, D, and F are omitted as none of the mice died during the experiments. The weights of all the groups are shown in Table I. It is seen that all the animals in Groups A, B, C, and D lost similar amounts.

The mice on the low protein diet plus methionine are the most susceptible to swine influenza, those on the low protein diet alone the least susceptible. The increased susceptibility of the mice receiving methionine over the other 2 groups is significant statistically. The differences between the mice on the Rock-

land Mouse Diet and on the low protein diet has been significant in some experiments but not in others.

Six experiments similar to the above and others along the same lines have given similar results. Cystine used under similar conditions increased slightly the susceptibility of the mouse to swine influenza. The effect of other amino acids is now being studied.

We have no explanation as to why methionine decreased the susceptibility of the rabbit to vaccinia and increased the susceptibility of the mouse to swine influenza. The experiments which we have in progress may elucidate this problem. Three possibilities appear now: (1) Methionine in some way lowers the resistance of the host to infection, possibly by increasing the permeability of the cell to the virus. (2) Methionine may interfere with the formation of antibodies. (3) Multiplication of the virus may be aided by methionine.

Conclusions. Mice on a low protein diet, if given methionine, have a greatly increased susceptibility to the swine influenza virus.

16291 P

Effect of Adrenalectomy Upon Level of Blood Amino Acids in the Eviscerated Rat.

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From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Following evisceration the concentration of amino acids in the blood increases gradually throughout the period of survival but this rise can be completely suppressed by the administration of optimal amounts of insulin with glucose.¹ In the present study it was

shown that adrenalectomy suppressed the level of blood amino acids below that of non-adrenalectomized eviscerated rats at each

¹ Ingle, D. J., Prestrud, M. C., and Nezamis, J. E., *Am. J. Physiol.*, 1947, **150**, 682.

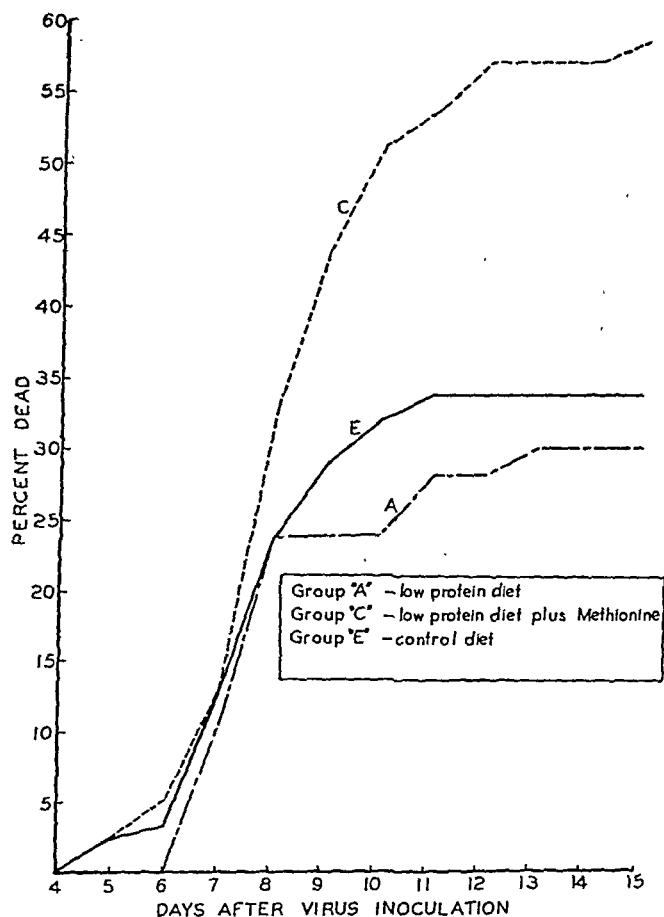


CHART 1.
The percentage of mice on different diets dying after injection with swine influenza.

essential that the substance to be studied should be added to a diet which by itself caused some malnutrition and increased resistance. A low protein diet was found to meet these requirements. Although malnutrition generally increases the animal's resistance to infection it does, if too extreme, result in the death of the animals from starvation. For this reason mice under similar conditions to those injected were kept as controls, in order to see if any of the deaths were due to malnutrition.

In the following experiments 360 male adult mice weighing approximately 25 g each were divided into 6 groups. The mice in Groups A, B, C, and D were fed the low protein diet, but in addition Groups C and D

were given daily intraperitoneal injections of 15 mg of an aqueous solution of methionine. After 2 weeks the mice in Groups A and C were inoculated intranasally, under ether anesthesia, with swine influenza virus.[†] The virus was diluted before inoculation to the 50% point, although similar results could be obtained with other strains of both human and swine influenza virus. This virus was used in most of the experiments, since the 50% point was more constant in various groups of mice. In these experiments it is

[†] The virus was kindly supplied to us by Dr. Joseph Beard of Duke University. It was an egg-adapted strain of swine influenza which came originally from Dr. R. E. Shope and has been designated as Strain 15.

Effects of B Vitamins, Yeast and Liver on Ovaries of Immature Rats Fed Massive Doses of Alpha-Estradiol.

B. H. ERSHOFF AND H. B. MCWILLIAMS.

From the Emory W. Thurston Laboratories, Los Angeles, California.

It is well established that massive doses of estrogens retard growth and inhibit gonadal development in the immature rat. Available data indicate that these effects may be counteracted, at least in part, by dietary means. Ershoff and Deuel¹ observed that the gonadal weight of immature rats fed massive doses of alpha-estradiol was significantly greater on diets containing yeast than on rations containing the B vitamins in synthetic form. In the present communication further data are presented on the effects of diet on ovarian development in the alpha-estradiol-fed rat.

Procedure and Results. Four basal rations were employed in the present experiment: diets A, B, C, and D. Diets A and B were purified rations containing the B-complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. All 4 rations were supplemented with 0.0 and 10.0 mg of alpha-estradiol per kg of diet.* Sixty-four female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 42.0 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad lib.* the diets listed in Table I. Feeding was continued for 8 weeks. Animals were autopsied on the 56th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

Results are summarized in Table II. In agreement with earlier findings¹ the effects of

alpha-estradiol feeding in the immature rat were dependent on the diet employed. On the synthetic ration (diet A₂) ovaries appeared infantile both in weight and microscopic appearance. Similar results were obtained in alpha-estradiol-fed rats receiving additional B vitamins or yeast (diets B₂ and C₂). In the whole liver series (diet D₂), however, ovarian weights averaged approximately twice the values obtained on other alpha-estradiol-containing rations; and histologically ovaries appeared normal in 8 of the 10 rats in this series. Growth was markedly reduced in all rats fed alpha-estradiol-containing rations. Gain in body weight was somewhat greater in the liver series (diet D₂) than on other rations employed, but with the possible exception of the A₂ series these differences were not significant. On alpha-estradiol-free rations body and ovarian weights did not differ significantly on any of the diets employed, and histologically ovaries appeared normal in all groups.

Discussion. It is becoming increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are dependent on the nutritional state and the diets employed. In acute deficiencies of essential nutrients it is readily recognized that resulting abnormalities in cellular metabolism may profoundly affect response to certain drugs. What is less well recognized, however, is that these drugs themselves may precipitate a deficiency state. Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine, and pantothenic acid,³ while dicumaryl, salicylates, and other drugs may precipi-

¹ Ershoff, B. H., and Deuel, H. J., Jr., *Am. J. Physiol.*, 1946, **145**, 465.

* The alpha-estradiol for these experiments was obtained from the Schering Corporation, Bloomfield, N.J. One mg alpha-estradiol \approx 12,000 R.U. or 120,000 I.U. estrone.

² Sure, B., *J. Nutrition*, 1941, **22**, 499.

³ Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, **135**, 474.

⁴ Shapiro, S., Redish, M. H., and Campbell, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 12.

level of insulin dose and glucose load.

Methods. Male rats of the Sprague-Dawley strain were eviscerated at 250 g weight. Adrenalectomy was performed in the same operation. Immediately following operation all of the animals were given continuous intravenous infusions of 0.9% sodium chloride solution with and without added glucose and crystalline zinc insulin (Lilly). The volume was 20 cc per 24 hours per rat. The details of the methods have been described.¹

Experiments and Results. Equal numbers of eviscerated rats and adrenalectomized-eviscerated rats were studied simultaneously. Samples of blood were taken from the abdominal aorta at the end of 24 hours. Sixteen pairs of rats were given 4 units of insulin and 40 mg of glucose per 100 g of rat per hour (40/100/h); 10 pairs were given 1 unit of insulin and 40/100/h; 16 pairs were given 0.5 units of insulin and 24/100/h; and 7 pairs were given no insulin and 8/100/h of glucose. At each level of insulin dosage and glucose load the average value for amino acids was lower in the adrenalectomized-eviscerated rats than in the eviscerated rats. Likewise, in each of the 49 pairs of rats the value for blood

amino acids was lower in the adrenalectomized rat than in the non-adrenalectomized rat. The data are summarized in Fig. 1.

Discussion. These results are consistent with an earlier study showing that insulin prevents the rise in blood amino acids which follows evisceration in the rat. The effect of adrenalectomy upon the level of blood amino acids in the eviscerated rat is apparently independent of insulin dosage and glucose load. Friedberg and Greenberg² have shown that the level of plasma amino acids is depressed by adrenalectomy in the non-eviscerated rat. Roberts³ reported that adrenalectomy in the eviscerated rat did not influence the rate of rise of blood amino acids but the periods of observation were shorter than in the present study and other conditions were different.

How do these data relate to the subnormal ability of the untreated adrenalectomized animal to mobilize its cellular proteins during stress? The level of blood amino acids in the eviscerated animal represents a balance between the breakdown and resynthesis of tissue proteins the rates of which were not appraised in this study. What would be the effect of cortical hormone overdosage upon the level of blood amino acids in eviscerated animals? Cortical hormone overdosage is antagonistic to the hypoglycemic effect of insulin in the normal force-fed rat⁴ and the eviscerated rat.⁵ Would it antagonize the effect of insulin in suppressing the rise in blood amino acids which follows evisceration? Further studies are planned.

Summary. Adrenalectomy in the eviscerated rat lowered the level of amino acids in whole blood at each level of insulin dosage and glucose load when determined 24 hours after operation.

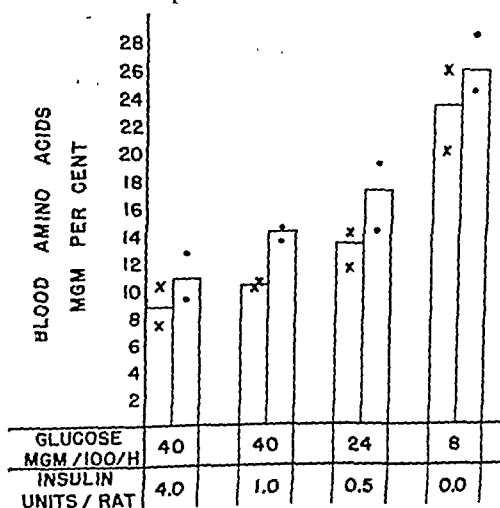


FIG. 1.

Level of blood amino acids at the end of 24 hours of continuous intravenous infusion. Averages and Range.

• Eviscerated rats.

x Adrenalectomized-eviscerated rats.

² Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, **168**, 405.

³ Roberts, S., *Endocrinology*, 1946, **30**, 80.

⁴ Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinology*, 1945, **37**, 341.

⁵ Ingle, D. J., Prestrud, M. C., Nezamis, J. E., and Kuizenga, M. H., *Am. J. Physiol.*, 1947, **150**, 423.

doses of strychnine, promin, dinitrophenol, sulfanilamide, atabrine, and other drugs have been recognized for years.⁸⁻¹¹ Similar results have been observed following toxic doses of diethylstilbestrol⁸ and desiccated thyroid.¹²⁻¹⁴ In the present experiment toxic effects of alpha-estradiol were similarly modified by dietary means.

Findings indicate that the effects of alpha-estradiol feeding in the immature rat were dependent on the diet employed. On the synthetic ration (diet A₂) ovaries appeared infantile both in weight and microscopic appearance. Similar results were obtained with animals fed additional B vitamins or yeast (diets B₂ and C₂). Oral administration of desiccated whole liver, however, resulted in a significant increase in ovarian weight with ovaries resembling histologically those of the normal rat. On alpha-estradiol-free rations ovaries did not differ significantly in weight or microscopic appearance on any of the diets employed. Available data indicate that the beneficial effects of liver on ovarian

development in the immature alpha-estradiol-fed rat were not due to any of the known B vitamins. This is indicated by the fact that ovaries remained infantile on diet B₂ although this ration contained all known members of the vitamin B complex in amounts exceeding their presence in the liver-containing diet D₂. It is suggested, therefore, that desiccated whole liver contains some factor(s) other than the known B vitamins whose requirement is increased following prolonged feeding of alpha-estradiol.[†] In previous work with smaller doses of alpha-estradiol yeast was also found to promote ovarian development in the alpha-estradiol-fed rat.¹ Such was not the case, however, under conditions of the present experiment.

Summary. Desiccated whole liver counteracted the inhibition of ovarian development observed in immature rats fed massive doses of alpha-estradiol. The protective factor in liver was distinct from any of the known members of the vitamin B complex. It was not present in significant amounts in yeast.

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[†] The unknown nutrient may be part of an enzyme system concerned with estrogen inactivation. It is possible that prolonged feeding of alpha-estradiol increased requirements for this factor on diets A₂, B₂, and C₂ to the point that a deficiency occurred, interfering with estrogen-inactivation and resulting in impaired secretion of pituitary gonadotropins.¹⁵

¹⁵ Zondek, B., *Clinical and Experimental Investigations on the Genital Functions and Their Hormonal Regulation*, Williams & Wilkins, Baltimore, 1941.

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H. S. RUBINSTEIN AND ALBERT A. KURLAND.

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In carrying out electro-shock therapy on psychotic patients it is frequently observed that some fail to immediately respond to the electrical stimulus with a major convulsive

seizure. At times, especially in attempting to establish the convulsive threshold for a particular patient, it becomes necessary to induce 3 or 4 minor responses before the

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A ₁ and A ₂	Diet B ₁ and B ₂	Diet C ₁ and C ₂	Diet D ₁ and D ₂
Yeast*	0.0	0.0	10.0	0.0
Whole liver powder†	0.0	0.0	0.0	10.0
Casein‡	22.0	22.0	22.0	22.0
Salt mixture§	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5

Alpha-estradiol was incorporated in diets A₂, B₂, C₂, and D₂ at a level of 10.0 mg per kg of diet.

To each kg of diet A, C, and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg, and choline chloride 1.2 g.

To each kg of diet B were added: thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, inositol 1.2 g, *p*-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-naphthoquinone 10 mg and choline chloride 1.2 g.

Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A-D concentrate|| containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* Brewer's Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

† Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

‡ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Sure's Salt Mixture No. 1.2

|| Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

TABLE II.
Effects of Alpha-estradiol on Body and Ovarian Weight in the Immature Rat.

Dietary Group	No. of animals	Initial body wt, g	Gain in body wt 8-wk period,* g	Avg ovarian wt,* mg
Alpha-estradiol Series.				
A ₂	10	42.8	97.9 ± 3.7	13.7 ± 1.1
B ₂	10	42.1	105.1 ± 6.6	16.1 ± 1.3
C ₂	10	41.9	103.4 ± 5.5	18.9 ± 1.4
D ₂	10	42.2	117.9 ± 3.6	37.2 ± 2.7
Control Series.				
A ₁	6	41.7	146.4 ± 9.0	47.0 ± 2.4
B ₁	6	42.0	152.3 ± 7.8	49.1 ± 3.0
C ₁	6	41.5	146.8 ± 6.9	43.2 ± 3.2
D ₁	6	41.7	169.7 ± 9.3	44.8 ± 2.1

* Including standard error of the mean calculated as follows: $\frac{\sqrt{\sum d^2}}{n} / \sqrt{n}$ where "d" is

the deviation from the mean and "n" is the number of observations.

tate a deficiency of vitamin K.⁴⁻⁶ In addition to the known nutrients, however, requirements for various unknown factors may also be increased following the administration of certain drugs. These "minor vitamins" are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the

intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient.⁷ Whole liver and yeast are potent sources of such unknown nutrients. The beneficial effects of these latter substances in animals inhaling carbon tetrachloride or fed toxic

⁵ Shapiro, S., *J. A. M. A.*, 1944, **125**, 546.

⁶ Collins, E. N., and Hoffman, A. D., *Cleveland Clin. Quart.*, 1943, **10**, 105.

⁷ Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

doses of strychnine, promin, dinitrophenol, sulfanilamide, atabrine, and other drugs have been recognized for years.⁸⁻¹¹ Similar results have been observed following toxic doses of diethylstilbestrol⁸ and desiccated thyroid.¹²⁻¹⁴ In the present experiment toxic effects of alpha-estradiol were similarly modified by dietary means.

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seizure. At times, especially in attempting to establish the convulsive threshold for a particular patient, it becomes necessary to induce 3 or 4 minor responses before the

desired major reaction becomes evident. In other patients, especially in those who are markedly disturbed, it has been observed that a single convulsion may fail to induce adequate sedation. The safety of repeated convulsions in such overly-disturbed patients has, therefore, been considered. Since a survey of the literature has failed to disclose the tolerance of animals to repeated electrically-induced convulsions and because of a fear of the consequences of repeated electrical stimulation this procedure had to be deferred.

Because of a need for such information it was deemed advisable to study this problem experimentally.

For this purpose 11 adult cats (exact ages unknown) were subjected to repeated electrical stimulation at approximately five-minute intervals. In 8 animals (Group A) this procedure was continued until each animal expired. The other 3 cats (Group B) were permitted to recover after having been subjected to a prolonged series of convulsions. On the basis of experience with patients submitted to electroshock this procedure was deemed entirely painless both at the time the convulsions were induced and after the convulsions were over.

The apparatus used to deliver the current was similar to that employed on patients. This utilized the 60-cycle 120-volt house current and consisted of a *step-up and step-down transformer* operated by an electrical timing switch. These were so designed that the duration of the current flow and voltage could be varied by the operator (Lektra Laboratories, New York). The lower voltage range was especially calibrated thus permitting a more accurate determination of the voltage required for the small animal used. The duration of the stimulus was maintained at a constant of 0.2 seconds. The electrodes used were especially constructed to better conform to the small animal head (Lektra Laboratories, New York). In each instance the convulsion was induced by the minimal convulsive dose and this had to be gradually increased in each animal as the experiment progressed.

Results. These have been tabulated in Tables I and II. From Table I it may be

TABLE I.
Tabulation of Data in Electrically-induced Convulsions in the Cat. Stimuli were repeated at 3.5-5.5-minute intervals and the duration of each stimulus was 0.2 seconds.

Cat Number	Group A (Expired)										Group B (Survived)			
	8	15*	16*	19	20*	21*	22*	24*	Avg	17*	18*	23		
Wt in lbs	7 3/4	5 1/4	4 1/4	4 3/4	4 3/4	6 1/2	5 3/4	5	58	8	7	5		
Major reactions	30	64	60	46	53	49	75	87	14.4	20	49	95		
Minor reactions	26	20	31	2	9	8	12	7	6.4	22	4	10		
Duration of experiment, hr and min.	4-40'	5-50'	6-56'	4-44'	5-23'	5-0'	7-40'	8-19'	0-4'	5-25'	5-17'	9-0'		
Initial voltage	80	90	50	40	40	40	40	40	52.5	50	50	40		
Terminal voltage	140	150	190	80	90	80	140	150	127.5	120	80	160		
Initial amperage (in ma.)	160	100	100	57	57	50	57	44	78.12	71	71	50		
Terminal amperage (in ma.)	166	333	475	133	103	177	311	300	278.25	228	160	355		
Coulombs (Q = It)	1.83	3.65	5.24	0.91	0.99	1.30	3.20	3.23	2.54	1.85	1.22	4.28		
Q/lbs	0.24	0.70	1.22	0.22	0.21	0.20	0.55	0.65	0.50	0.23	0.17	0.85		

* Brain material available.

A Biological Method for Determination of Curare and Erythroidine-Alkaloids.

E. P. PICK AND G. V. RICHARDS.

From the Merck Institute for Therapeutic Research, Rahway, N.J., and the Laboratories of the Mount Sinai Hospital, New York City.

Recent studies¹ have shown that mice pretreated with morphine sulfate are especially sensitive to curare and erythrina-alkaloids. Since these alkaloids in microgram amounts antagonized both excitement and the characteristic Straub-phenomenon² on the tails of morphinized mice before any peripheral paralyzing action became evident, it was of interest to investigate whether this procedure could be employed as a sensitive biological method for the determination of low concentrations of these alkaloids.

Two methods which permit the estimation of curare *in vitro* have been developed which make use of isolated mammalian preparations: one method (Bülbring-Chou³) employs the phrenic nerve diaphragm preparation from the rat and tests the curare depressant effect of rapid motor nerve stimulation; the other (P. D. Garcia de Jalon⁴) is based on the well known antagonistic action of curare⁵ on the acetylcholine contracture in *M. rectus abdominis* of the frog. These *in vitro* methods provide a rapid check on pure curare preparations, but are less useful for the exact measurement of impure preparations and tissue extracts. The rabbit head drop method of McIntyre and Holaday⁶ provides an accurate *in vivo* assay for this type of preparation but

requires the use of an appreciable amount of substance. Recently Skinner and Young⁷ have proposed a mouse assay which is dependent on the peripheral paralyzing action of the alkaloids as determined in the sloping rotating cylinder apparatus of Young and Lewis.⁸

Method. Groups of 5 to 10 white mice, weighing 18-20 g each, are injected subcutaneously with 0.5 mg of morphine sulfate. Restlessness and the typical tail reflex occur generally within 5 to 10 minutes and persist for 2 hours or more. Doses smaller than 0.5 mg of morphine sulfate are not recommended because the characteristic morphine effect is either delayed or of not sufficient duration. Curare and erythrina preparations, dissolved in 0.85% sodium chloride, are injected intraperitoneally in a volume not exceeding 0.5 cc into animals exhibiting the typical morphine reaction. A positive effect appears within 5 to 10 minutes and is characterized by disappearance of the excitement phenomena and relaxation of the tails. This period lasts usually for 10-15-25 minutes and is followed by the gradual reappearance of restlessness and tail phenomenon. At this point, the mice may again be used for testing without danger of alteration of the alkaloid response since intraperitoneally injected curare is rapidly destroyed or excreted.

Table I shows the results obtained following the injection of graded doses of various curare and erythroidine preparations. From these data it can be determined that the median effective dose and its standard error of *d*-tubocurarine chloride is $2.8 \pm 0.2 \mu\text{g}$;

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they were experienced in such concentrated form. It is interesting that the oldest appearing cat in the group (No. 8) was first to die. The brain of this animal, like those of all others, showed no evidence of gross pathology. Microscopic studies will be reported later. Electroencephalographic changes were observed, however, as the experiment progressed and these consisted for the most part of a gradual slowing and decrease in voltage. Detailed study of these findings is now under way.

The rise of the convulsive threshold as the experiment progresses is also significant in that it is at first very gradual. Later it increases in accelerated fashion so that in some cases the animal requires 4 times the initial voltage and as much as 7 times the initial amperage before responding with a convulsion. This is probably due to exhaustion, and experiments are now being planned to verify this hypothesis.

The striking fact obtained from this study is that no positive correlation between the amount of electricity applied and the tolerance of the animal could be demonstrated. This lack of correlation can best be appreciated from the tabulated coulomb-body weight ratios (Q/lbs) in Table I. These ratios show a variation of from 0.21 to 1.22 total coulombs per pound body weight in those animals which expired; while the surviving animals showed variations in ratios which were almost as great. This seems to indicate that of those cats which expired some required almost 6 times as many coulombs as others. It, therefore, appears that tolerance of electrical stimulation by a particular animal varies for each animal as an individual. Perhaps the best criterion of the ability of the cat to withstand electrical stimulation is illustrated in Table II. Here, a comparison of the original convulsive quantity of current (Q_2) with the total coulombs used (Q_1) discloses that in those cats expiring this ratio varied from 57 to 403. In the 3 surviving

cats these ratios were 57, 87, and 428 respectively. In other words, of all the cats used it required at least 57 times the minimal convulsive coulomb value before any of them died. And in one instance a cat survived 428 times the original minimal convulsive coulomb value.

Summary. Adult cats submitted to repeated stimulations with, whenever possible, minimal convulsive doses of electricity showed an ability to withstand 30 to 87 major convulsions before expiring. Of those cats surviving the experiment one experienced 95 such convulsions. Initial voltage was observed to rise in one instance to approximately 4 times, and initial amperage was observed in one instance to rise to approximately 7 times the original minimal convulsive levels as the experiment progressed. The oldest appearing cat in the group was first to succumb. The brain of this cat, like the brains of all others, showed no gross pathological changes. Electroencephalographic changes in the form of gradual slowing and decreasing voltage were observed as the experiment progressed. The surviving cats showed no gross defects in behavior 21 days after the last convulsion.

It is assumed that death when it occurred was the result of exhaustion. No correlation between total applied current and tolerance could be ascertained. The striking observation is the unexpectedly high tolerance of the adult cat for repeated minimal convulsive-inducing doses of electricity. This is quantitatively best shown in comparisons between original minimal convulsing coulomb values and total coulombs used. In those cats which expired this total varied from 57 to 403 times the original minimal convulsive quantity. Of the surviving cats one received as much as 428 times the original minimal convulsive coulomb value. These experiments, therefore, point to the relative safety of the minimal convulsive dose of electricity in the cat and suggest a probable greater tolerance in the human than has heretofore been recognized.

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TABLE I.
Graded Doses of Various Curare and Erythroidine Preparations Antagonizing the Effect of Morphine Poisoning on Mice.

Drug	Dose per 20 g mouse*	No. of animals	No. of animals responding with disappearance of morphine symptoms	Duration of curare effect in min.
Cryst. <i>d</i> -tubocurarine chloride	2.5 μ g	10	5	5-10
	3.0 "	15	10	5-13
	5.0 "	10	10	25-50
Strychnos curare (Merck)	20.0 "	5	0	—
	30.0 "	5	5	12
	40.0 "	5	5	24
Cryst. dihydro- β -erythroidine-bromide	40.0 "	5	0	—
	50.0 "	10	10	18
	60.0 "	10	10	10
Intocostrin (Squibb)	15.0 milliunits	5	—	—
	20.0 "	10	5	26
	25.0 "	10	10	19
	30.0 "	10	10	17-35

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

TABLE II.
Doses of Various Alkaloids Influencing the Effect of Morphine Poisoning on Mice.

Drug	Dose in γ per 20 g mouse*	Effect on morphine excitement and tail phenomenon
Quinine methochloride	100	partial
" ethochloride	500	no effect
" sulfate	500	" "
Tetramethylammonium chloride	100	" "
	200	complete effect
Tetraethylammonium "	114	partial effect
Cryst. thiamine hydrochloride	500	no effect
	1000	complete effect
Acetylcholine "	50	no effect
Histamine "	300	" "
	400	complete toxic effect
Nicotine base	75	no effect
	100	partial toxic effect
Guanidine hydrochloride	1000	no effect
Bulbocapnine "	100	partial effect
	250	partial effect, quiet, tails erect
Scopolamine hydrobromide	100	no effect
	250	partial effect (excited)
	500	complete effect

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

for Strychnos Curare (Merck), 24.0 ± 2.0 μ g; for dihydro- β -erythroidine bromide, 44.0 ± 3.0 μ g; and for Intocostrin (Squibb), 20.0 ± 2.0 milliunits.

Several other drugs have been tested for their ability to antagonize morphine excitement and tail phenomenon. These results

are shown in Table II. None of these drugs completely antagonize the effects of morphine poisoning in mice in doses smaller than 100 γ per 20 g mouse. Thiamine hydrochloride, quinine ethochloride, quinine sulfate, histamine hydrochloride, bulbocapnine hydrochloride, scopolamine hydrobromide, and guanidine

hydrochloride were without effect in doses of 400-1000 γ . Nicotine base exerted a partial effect in 100 γ dose; histamine was effective only in toxic amounts of 400 γ and acetylcholine chloride was ineffective in a dose of 50 γ . This analysis shows that although the several quaternary alkaloids and other drugs mentioned in Table II may influence the synaptic transmission or the myoneural junction, they do not compare with curare and erythroidine in their influence on the outward manifestation of morphine poisoning in mice. It may be noted here that Myanesin⁹ [α,β -dihydroxy- γ -2-(methylphenoxy-propane)] in doses of 8 mg per mouse despite its curare-like action on the striated muscles, is not able to depress the tail reflex in morphinized mice. The data

seem to indicate that because of the relatively small amounts of alkaloid required to produce a positive effect, this method may be useful for the determination of curare or erythroidine in tissue extracts or urine even though other active substances may be present.

Summary. A biological method for qualitative and quantitative determination of curare and erythroidine is described. The method depends upon the antagonistic action of these alkaloids on the excitement and tail phenomenon in morphine-poisoned mice. The median effective dose and standard error for crystalline *d*-tubocurarine chloride is $2.8 \pm 0.2 \mu\text{g}$; for Strychnos Curare (Merck), $24.0 \pm 2.0 \mu\text{g}$; for dihydro- β -erythroidine bromide, $44.0 \pm 3.0 \mu\text{g}$; and for Intocostin (Squibb), 20.0 ± 2.0 milliunits. Far higher doses of other drugs, including quaternary alkaloids, are necessary to antagonize the effect of small amounts of morphine in mice.

⁹ Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, 1, 265.

16295

Effect of Anoxic Anoxia on Propulsive Motility of the Small Intestine.*

E. J. VAN LIERE, W. V. CRABTREE, D. W. NORTHUP, AND J. C. STICKNEY.

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It has been shown that although anoxic anoxia does not significantly affect the propulsive motility of the small intestine of the dog, it does so in the mouse.¹ Since there is this apparent specific difference it was deemed worthwhile to study the effect of anoxic anoxia on still a different species, namely, the rat.

Methods. Paired albino rats as nearly alike in weight and age as possible were fasted 24 hours. One served as a control and the other was subjected to anoxic anoxia. (In all, 42 control and 42 experimental animals were used.) The following partial pressures of oxygen were employed: 94 mm Hg, 80 mm

Hg, 63 mm Hg, and 53 mm Hg; corresponding to simulated altitudes of 14,000, 18,000, 24,000, and 28,000 feet.

The experimental animal was given 2 cc of a charcoal-acacia mixture by stomach tube, and after allowing 10 minutes for some of this mixture to enter the small intestine, placed into a low-pressure chamber for 30 minutes. On removal from the chamber it was decapitated and the small intestine removed. The latter was slit open and the distance the charcoal-mixture had traversed the small intestine was measured. Control intubated animals were maintained at atmospheric pressure; in order to keep other experimental conditions as nearly alike as possible, they were placed in cages on top of the low-pressure chamber, so that they were exposed to the same noise and vibration as the experi-

* Aided by a grant of the Ella Sachs Plotz Foundation.

¹ Van Liere, E. J., Northup, D. W., Stickney, J. C., and Emerson, G. A., *Am. J. Physiol.*, 1943, 140, 119.

TABLE I.
Graded Doses of Various Curare and Erythroidine Preparations Antagonizing the Effect of Morphine Poisoning on Mice.

Drug	Dose per 20 g mouse*	No. of animals	No. of animals responding with disappearance of morphine symptoms	Duration of curare effect in min.
Cryst. <i>d</i> -tubocurarine chloride	2.5 μ g	10	5	5-10
	3.0 "	15	10	5-13
	5.0 "	10	10	25-50
Strychnos curare (Merck)	20.0 "	5	0	—
	30.0 "	5	5	12
	40.0 "	5	5	24
Cryst. dihydro- β -erythroidine-bromide	40.0 "	5	0	—
	50.0 "	10	10	18
	60.0 "	10	10	10
Intocostrin (Squibb)	15.0 milliunits	5	—	—
	20.0 "	10	5	26
	25.0 "	10	10	19
	30.0 "	10	10	17-35

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

TABLE II.
Doses of Various Alkaloids Influencing the Effect of Morphine Poisoning on Mice.

Drug	Dose in γ per 20 g mouse*	Effect on morphine excitement and tail phenomenon
Quinine methochloride	100	partial
" ethochloride	500	no effect
" sulfate	500	" "
Tetramethylammonium chloride	100	" "
	200	complete effect
Tetraethylammonium "	114	partial effect
Cryst. thiamine hydrochloride	500	no effect
	1000	complete effect
Acetylcholine "	50	no effect
Histamine "	300	" "
	400	complete toxic effect
Nicotine base	75	no effect
	100	partial toxic effect
Guanidine hydrochloride	1000	no effect
Bulbocapnine "	100	partial effect
	250	partial effect, quiet, tails erect
Scopolamine hydrobromide	100	no effect
	250	partial effect (excited)
	500	complete effect

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

for Strychnos Curare (Merck), 24.0 ± 2.0 μ g; for dihydro- β -erythroidine bromide, 44.0 ± 3.0 μ g; and for Intocostrin (Squibb), 20.0 ± 2.0 milliunits.

Several other drugs have been tested for their ability to antagonize morphine excitement and tail phenomenon. These results

are shown in Table II. None of these drugs completely antagonize the effects of morphine poisoning in mice in doses smaller than 100 γ per 20 g mouse. Thiamine hydrochloride, quinine ethochloride, quinine sulfate, histamine hydrochloride, bulbocapnine hydrochloride, scopolamine hydrobromide, and guanidine

TABLE I.
Ration Fed to Each Group of Rats.*

Ingredient	Groups					
	1	2	3	4	5	6
Casein, Labco	5	5	10	15	20	30
PGA	0.4 mg/100 g	0	0	0	0	0
Glucose	83	83	78	73	68	58
Cod Liver Oil	2	2	2	2	2	2
Salt Mixture 446	4	4	4	4	4	4
Corn Oil	4	4	4	4	4	4
Sulfathalidine	2	2	2	2	2	2

* The following vitamins were added per 100 g basal ration:

Thiamine	mg
Riboflavin	.25
Pyridoxine	.25
Nicotinic Acid	1.0
Calcium-pantothenate	2.0
Biotin	.01
p-Aminobenzoic Acid	5.0
Inositol	10.0
Choline	100.0
2-methyl-1,4-naphthoquinone	0.1
Vit. A	{ Haliver oil
Vit. D	{ 2 drops/rat/week
α -Tocopherol	1 mg/rat/week

diet of the rat is a significant factor in the production of the blood dyscrasias typical of PGA deficiency. In order to determine what level of protein might be fed and still produce

anemia on a PGA-deficient ration containing sulfathalidine, the influence of different casein levels and the effect of PGA intake on anemia production in growing rats were investigated.

Experimental. Six groups of weanling rats, 5 in each group, were fed the rations given in Table I. The rats were fed *ad libitum* and kept on experiment for six weeks. Their growth curves are given in Fig. 1. Starting with the third week on the experiment, blood hemoglobin levels and red blood cell counts were determined weekly for the next 4 weeks. The hemoglobin values are given in Fig. 2. The red blood cell counts at 6 weeks are given in Table II.

Discussion. From this experiment it appears that the anemia produced by a protein deficiency on a sulfathalidine-containing PGA-low ration will respond to either PGA or to protein (Fig. 1 and Table IV). On the other hand, the lack of growth on a 5% casein diet was not affected by the addition of PGA to this diet (Fig. 2 and Table III).

The differences between the average gains of the rats for each group were analyzed statistically and no difference was found be-

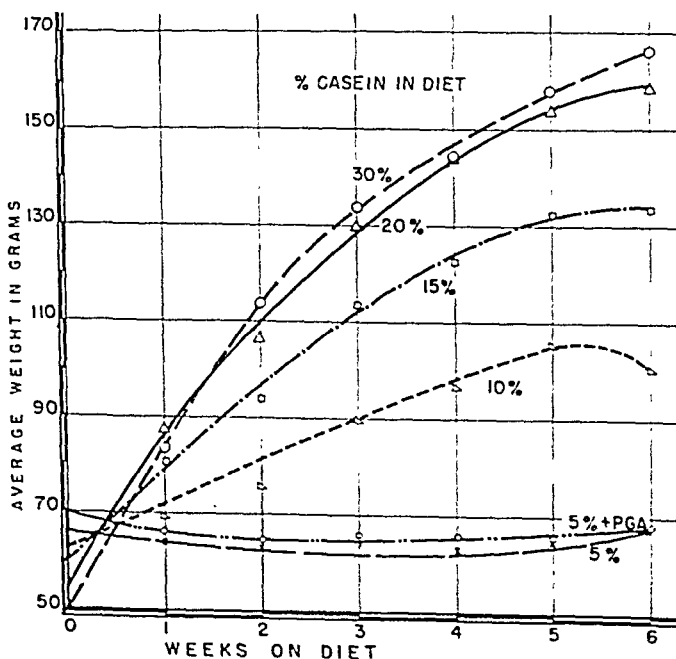


FIG. 1.
Growth curves of experimental rats.

TABLE I.
Effect of Anoxia on Peristalsis of the Small Intestine in Rats.

PO ₂ , mm Hg	Altitude, feet	Control		Anoxia		"P"*
		No. of animals	% of gut traversed at end of 40 min.	No. of animals	% of gut traversed at end of 40 min.	
94	14,000	11	71	11	64	0.19
80	18,000	9	82	11	64	<0.01
63	24,000	10	76	10	50	<0.001
53	28,000	12	78	10	40	<0.001

* Significant when "P" (according to Fisher) is 0.05 or less.

mental animals.

Results and Discussion. The data, Table I, show that anoxic anoxia did not decrease the propulsive motility of the small intestine significantly at a simulated altitude of 14,000 feet. Above this level, however, the motility was significantly decreased by the anoxia.

The results indicated that the threshold for anoxia on the peristalsis of the small intestine lay between a simulated altitude of 14,000 feet and 18,000 feet. In previous work¹ it was shown that the propulsive motility of the intestine of the mouse was not significantly decreased until a simulated altitude of 18,000 feet was reached. The results obtained with rats, therefore, show fair agreement with those for mice. Since anoxic anoxia stimulates the sympathetic division of the autonomic nervous system it would be expected that intestinal movements would be decreased.

The authors still can offer no adequate explanation for the negative effect of anoxic anoxia on the propulsive motility of the small

intestine in the dog. The current experiments suggest that as far as the propulsive motility of the small intestine is concerned the rat is somewhat less resistant to anoxia than the dog. However, it should be pointed out that both of these animals are relatively resistant to anoxic anoxia so that the difference observed by us between the two species probably cannot be attributed entirely to this factor.

Summary. The effect of anoxic anoxia on the propulsive motility of the small intestine of the albino rat was studied at the following partial pressures of oxygen: 94 mm Hg, 80 mm Hg, 63 mm Hg, and 53 mm Hg; corresponding to simulated altitudes of 14,000, 18,000, 24,000, and 28,000 feet. At a simulated altitude of 18,000 feet or higher there was a significant decrease in propulsive motility. The threshold lay between 14,000 and 18,000 feet. The results differ from those obtained in the dog but show fair agreement with those in the mouse.

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Protein Level and Pteroylglutamic Acid Intake on Growth Rate and Hemoglobin Level in the Rat.

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In attempts to produce a pteroylglutamic acid (PGA) deficiency in the pig, baby pigs were fed a PGA-low "synthetic" diet containing 30% casein and 2% sulfathalidine. However, no blood dyscrasias were observed.¹ Kornberg, Daft and Sebrell² and Daft³ have

shown that limitation of the casein in the

¹ Johnson, B., Connor, James, Marian, F., and Krider, J. L., *J. Animal Science*, 1947, **6**, 486.

² Kornberg, Arthur, Daft, Floyd S., and Sebrell, W. H., *Science*, 1946, **103**, 646.

³ Daft, Floyd S., *Fed. Proc.*, 1947, **6**, 405.

TABLE IV.
Statistical Treatment of Data. Hemoglobin Levels in 100 g/100 cc of Rats at 6 Weeks.

Group No.	1 5% + PAGA casein	2 5% casein	3 10% casein	4 15% casein	5 20% casein	6 30% casein
Rat 2	13.1	8.0	8.3	12.0	12.0	12.4
3	12.4	8.3	11.0	10.7	11.2	14.2
4	13.8	8.9	11.2	10.6	12.4	13.1
5	12.7	8.9	11.0	12.4	13.8	12.4
Mean	13.0	8.5	10.4	11.4	12.4	13.0
Group compared		1 vs. 2	2 vs. 3	3 vs. 4	4 vs. 5	5 vs. 6
Probability of Difference*		<.01	.025	.15	.12	.2
		1 vs. 3	2 vs. 4	3 vs. 5	4 vs. 6	
		<.01	<.01	.025	.025	
		1 vs. 4	2 vs. 5	3 vs. 6		
		.01	<.01	.01		
		1 vs. 5	2 vs. 6			
		.15	<.01			
		1 vs. 6				
		.028				

* Student's *t*.

of a more complete list of vitamins (including para-aminobenzoic acid, inositol, etc.) in our ration, to differences in carbohydrate, etc.; or it may be that sulfathalidine is not as useful for prevention of intestinal synthesis of PGA in nutritional experiments as are some of the other insoluble sulfa drugs. However, Teply, Krehl, and Elvehjem¹⁰ have produced growth depression on a 15% casein "synthetic" diet containing 78% sucrose and 2% sulfathalidine, which was partially corrected by feeding a "folic acid" concentrate. Wright, Skeggs,

and Sprague,¹¹ using diets containing 5% sulfasuxidine, have shown that on a high protein (30% casein) diet, "folic acid" stores in the liver and "folic acid" excretion in the feces are higher than on a normal (18% casein) diet. They also obtained better growth on the high protein diet, due to the lower PGA requirement.

Summary. Anemia was produced in growing rats by feeding a ration containing 5% casein and 2% sulfathalidine, to which no pteroylglutamic acid was added. This anemia was prevented either by the addition of pteroylglutamic acid to the diet or by increasing the casein to 20%.

There was no improvement in growth when PGA was added to the 5% casein diet, while 30% casein gave normal growth.

The sulfathalidine used in this experiment was very generously supplied by Sharp and Dohme, Philadelphia, Pa., through the courtesy of Dr. S. F. Scheidty.

¹¹ Wright, L. D., Skeggs, H. R., and Sprague, L. L., *J. Nutrition*, 1945, 29, 431.

⁴ Student, *Biométrica*, 1908, 6, 1.

⁵ Kornberg, A., Daft, F. S., and Sebrell, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1945, 58, 46.

⁶ Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, 98, 20.

⁷ Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F., *J. Biol. Chem.*, 1943, 148, 721.

⁸ Nielsen, E., and Elvehjem, C. S., *J. Biol. Chem.*, 1942, 145, 713.

⁹ Spieer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Publ. Health Rep.*, 1942, 57, 1559.

¹⁰ Teply, L. J., Krehl, W. A., and Elvehjem, C. A., *Am. J. Physiol.*, 1947, 148, 91.

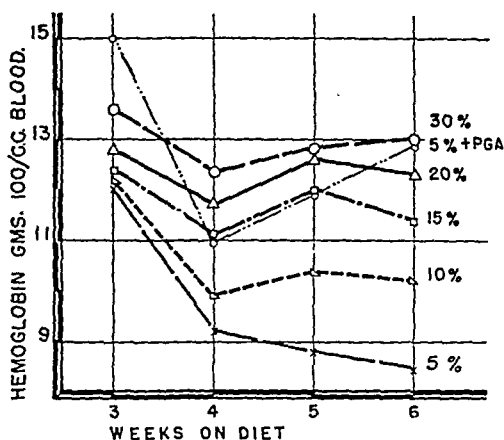


FIG. 2.

Hemoglobin concentration of blood from experimental rats.

TABLE II.

Average Red Blood Cell Count at 6 Weeks for Groups of Rats Fed Increasing Levels of Casein.

Group No.	%	Ration	Avg RBC millions per mm ³
1	5	Casein + PGA	7.16
2	5	Casein	5.11
3	10	"	5.95
4	15	"	6.22
5	20	"	6.60
6	30	"	7.15

tween those receiving 20 and 30% protein ($P = 0.45$), but significantly lower rates of gain were found at lower casein levels (30%

or 20% versus any other, $P < .01$). The statistical analysis of this data is given in Table III.

Statistical analysis of the data on concentration of hemoglobin in the blood at the end of six weeks, given in Table IV indicated: (1) that the presence of either PGA or of 15, 20, or 30% casein in the diet produced a significantly greater concentration of hemoglobin in the blood than did a 5% casein diet; (2) that diets containing 5% casein plus PGA or 20 or 30% casein produced equal hemoglobin concentrations; and (3) that without PGA, 10% casein produced no significantly greater hemoglobin concentration than did 5% casein. This would indicate that for normal hemoglobin formation in the absence of PGA, over 15% casein is required in the diet, that is, between 15 and 20% casein provides adequate precursors for the synthesis of PGA.

It is also to be noted that even with 2% sulfathalidine in the diet, no significant anemia was observed in the absence of PGA on a 20% casein diet. Several workers⁵⁻⁹ have produced typical symptoms of PGA deficiency using 18% casein diets with various sulfa drugs used as the intestinal bacteriostatic agents. Our inability to produce blood discrasias at 20% casein may be due to differences in the PGA content of Labco casein, to the inclusion

TABLE III.
Statistical Treatment of Data. Gains of Rats at 6 Weeks (g).

Group No.	1 5% casein + PGA	2 5% casein	3 10% casein	4 15% casein	5 20% casein	6 30% casein
Rat 1	1	8	32	74	126	125
2	-3	2	42	92	95	138
3	-3	1	34	74	108	111
4	-4	-3	52	55	105	104
5	dead	-1	31	82	95	108
Mean	-2.2	1.4	38.2	75.4	105.8	117.2
Rations compared		1 vs. 2	2 vs. 3	3 vs. 4	4 vs. 5	5 vs. 6
Probability of Difference*		.45	<.01	<.01	<.01	.45
		1 vs. 3	2 vs. 4	3 vs. 5	4 vs. 6	
		<.01	<.01	<.01	<.01	
		1 vs. 4	2 vs. 5	3 vs. 6		
		<.01	<.01	<.01		
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* Student.⁴

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of a more complete list of vitamins (including para-aminobenzoic acid, inositol, etc.) in our ration, to differences in carbohydrate, etc.; or it may be that sulfathalidine is not as useful for prevention of intestinal synthesis of PGA in nutritional experiments as are some of the other insoluble sulfa drugs. However, Tepley, Krehl, and Elvehjem¹⁰ have produced growth depression on a 15% casein "synthetic" diet containing 78% sucrose and 2% sulfathalidine, which was partially corrected by feeding a "folic acid" concentrate. Wright, Skeggs,

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Summary. Anemia was produced in growing rats by feeding a ration containing 5% casein and 2% sulfathalidine, to which no pteroylglutamic acid was added. This anemia was prevented either by the addition of pteroylglutamic acid to the diet or by increasing the casein to 20%.

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¹¹ Wright, L. D., Skeggs, H. R., and Sprague, L. L., *J. Nutrition*, 1945, 29, 421.

⁴ Student, *Biometrika*, 1908, 6, 1.

⁵ Kornberg, A., Daft, F. S., and Sebrell, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1945, 58, 46.

⁶ Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, 98, 20.

⁷ Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F., *J. Biol. Chem.*, 1943, 148, 721.

⁸ Nielsen, E., and Elvehjem, C. S., *J. Biol. Chem.*, 1942, 145, 713.

⁹ Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Publ. Health Rep.*, 1942, 57, 1559.

¹⁰ Tepley, L. J., Krehl, W. A., and Elvehjem, C. A., *Am. J. Physiol.*, 1947, 148, 91.

Effect of Vagotomy on Gastric Secretory Response to Histamine.*

HARRY A. OBERHELMAN, JR., AND LESTER R. DRAGSTEDT.

From the Department of Surgery of the University of Chicago.

During the course of routine studies on the gastric physiology of ulcer patients, it was noted that the gastric secretory response to a standard dose of histamine was decidedly less after complete gastric vagotomy than had been secured in the preoperative period. This surprising finding, which suggested that the physiological action of histamine was not solely upon the cellular elements in the gastric mucosa, prompted the present investigation. Early in the work on histamine, it was reported by Popielski¹ and by Keeton, Luckhardt, and Koch² that this drug could produce a gastric secretory response after section and subsequent degeneration of the vagus nerves to the stomach. Ivy and Javois³ found that histamine would stimulate gastric secretion in a denervated gastric pouch prepared after the method of Bickel, and Klein⁴ reported that a similar response could be obtained from a transplanted stomach pouch that had been deprived of its muscular layers and the myenteric plexus. These findings indicate that histamine can stimulate the peripheral neuroglandular mechanism of the stomach, or possibly the gland cell itself. Most investigators agree that histamine acts more intensely on acid producing cells than on the cells which elaborate pepsin. It has been suggested that the increased gastric secretion produced by histamine is secondary to the vaso-dilatation and increased blood supply to the mucosa produced by the drug. A central action of histamine is not indicated. The material presented in this paper includes studies both on

experimental animals and man.

Experimental Data. The stomach of was isolated from continuity with the alimentary tract by the method described by Dragstedt and Ellis.⁵ Great care was taken not to interfere with the vagus nerve supply and blood supply to the isolated stomach. A gold-plated cannula was introduced for the collection of gastric secretions and the continuity of the alimentary tract was restored by anastomosing the lower end of the esophagus to the upper end of the duodenum. Animals prepared in this fashion were found to secrete large volumes of gastric juice daily and they were kept in electrolyte and fluid balance by the injection of large amounts of salt solution intravenously. When they had entirely recovered from the surgical procedure, which usually required approximately two weeks, they were used for the following studies. The isolated stomach preparation of this type permits the quantitative collection of the gastric secretion from the entire stomach for any desirable period of time. After a control collection of the gastric secretion, one milligram of histamine phosphate was injected subcutaneously and the gastric juice secreted in the following 75 minutes examined for volume, free hydrochloric and total acid secretion. Usually a series of 4 such tests were performed on each animal to determine the average secretory response to histamine. A transthoracic section of the vagus nerves to the stomach was then performed under ether anesthesia with a positive pressure apparatus. The completeness of the vagus section was confirmed in each case by a negative response to insulin hypoglycemia. Following recovery from the vagus section, the animals were tested with histamine exactly as before the operation. The results obtained are indicated in Table I. It is apparent that the volume

* This work has been aided by a grant from the Douglas Smith Foundation for Medical Research, University of Chicago.

¹ Popielski, L., *Pflug. Arch. f. d. ges. Physiol.*, 1920, **178**, 214.

² Keeton, R. W., Luckhardt, A. B., and Koch, F. C., *Am. J. Physiol.*, 1920, **51**, 469.

³ Ivy, A. C., and Javois, A. J., *Am. J. Physiol.*, 1924, **71**, 604.

⁴ Klein, E., *Arch. Surg.*, 1932, **25**, 442.

⁵ Dragstedt, L. R., and Ellis, J. C., *Am. J. Physiol.*, 1930, **93**, 407.

TABLE I.
Effect of Vagotomy on Gastric Secretory Response to Histamine in Total Pouch Dogs.

Dog No.	Gastric juice secreted in 75 min. after the subcutaneous injection of 1 mg of histamine phosphate				
	Before vagotomy		After vagotomy		Decrease, %
	Vol., cc	Total HCl output m. eq.	Vol., cc	Total HCl output m. eq.	
865	162	20.7	81	7.9	50
875	139	17.8	45	4.1	67
898	78	8.72	44	3.42	77
					44
					60

Note: These data present the average of at least 4 tests on each dog.

TABLE II.
Effect of Atropine on Gastric Secretory Response to Histamine Before and After Vagotomy in Total Pouch Dogs.

Dog No.	Stimulus	Before vagotomy		After vagotomy	
		Vol., cc	HCl output, m. eq.	Vol., cc	HCl output, m. eq.
865	1 mg histamine	162	20.7	81	7.9
	2 mg atropine, 30 min. later 1 mg histamine	20	1.4	17	1.2
875	1 mg histamine	139	17.8	45	4.1
	2 mg atropine, 30 min. later 1 mg histamine	24	2.1	19	1.4

Note: These data present the average of at least 2 tests before and after atropine administration in each dog.

of gastric juice secreted in response to the subcutaneous injection of a standard dose of histamine was decreased by 50, 67, and 44% respectively, by complete division of the vagus nerves to the stomach. The total hydrochloric acid output in the gastric juice in response to histamine was diminished after vagotomy by 62, 77, and 60% respectively, as compared with preoperative values. Similar results were obtained in 2 additional animals prepared in the same way. In 2 of the animals, the effect of atropine on the secretion of gastric juice produced by the subcutaneous injection of histamine was determined both before and after section of the vagus nerves to the stomach. The results are indicated in Table II. One milligram of histamine phosphate was injected subcutaneously and the gastric secretion collected for a period of 75 minutes. Two milligrams of atropine sulphate were then given subcutaneously, and 30 minutes later, a second subcutaneous injection of one milligram of histamine phosphate was made. Following the

administration of the atropine, the volume of gastric juice secreted in response to the injection of the standard dose of histamine was reduced in the one case by 88, and in the other by 82%. Likewise, the total acid output decreased 93 and 88% respectively. It is interesting that following vagotomy, atropine exerted as great an inhibition to the gastric secretory response to histamine as before vagus section.

Clinical Studies. The studies on man were carried out on 2 groups of patients. The first comprised 15 patients with peptic ulcer who were treated by transthoracic section of the vagus nerves to the stomach at the Albert Merritt Billings Hospital, while the second group included 18 ulcer patients who underwent transabdominal gastric vagotomy at the Illinois State Penitentiary at Stateville. None of the patients in either group had an associated gastroenterostomy. In the first group, the observations were made as follows: Continuous aspiration of the gastric content was made during the night by means of an indwell

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During the course of routine studies on the gastric physiology of ulcer patients, it was noted that the gastric secretory response to a standard dose of histamine was decidedly less after complete gastric vagotomy than had been secured in the preoperative period. This surprising finding, which suggested that the physiological action of histamine was not solely upon the cellular elements in the gastric mucosa, prompted the present investigation. Early in the work on histamine, it was reported by Popielski¹ and by Keeton, Luckhardt, and Koch² that this drug could produce a gastric secretory response after section and subsequent degeneration of the vagus nerves to the stomach. Ivy and Javois³ found that histamine would stimulate gastric secretion in a denervated gastric pouch prepared after the method of Bickel, and Klein⁴ reported that a similar response could be obtained from a transplanted stomach pouch that had been deprived of its muscular layers and the myenteric plexus. These findings indicate that histamine can stimulate the peripheral neuroglandular mechanism of the stomach, or possibly the gland cell itself. Most investigators agree that histamine acts more intensely on acid producing cells than on the cells which elaborate pepsin. It has been suggested that the increased gastric secretion produced by histamine is secondary to the vaso-dilatation and increased blood supply to the mucosa produced by the drug. A central action of histamine is not indicated. The material presented in this paper includes studies both on

experimental animals and man.

Experimental Data. The stomach of dogs was isolated from continuity with the alimentary tract by the method described by Dragstedt and Ellis.⁵ Great care was taken not to interfere with the vagus nerve supply and blood supply to the isolated stomach. A gold-plated cannula was introduced for the collection of gastric secretions and the continuity of the alimentary tract was restored by anastomosing the lower end of the esophagus to the upper end of the duodenum. Animals prepared in this fashion were found to secrete large volumes of gastric juice daily and they were kept in electrolyte and fluid balance by the injection of large amounts of salt solution intravenously. When they had entirely recovered from the surgical procedure, which usually required approximately two weeks, they were used for the following studies. The isolated stomach preparation of this type permits the quantitative collection of the gastric secretion from the entire stomach for any desirable period of time. After a control collection of the gastric secretion, one milligram of histamine phosphate was injected subcutaneously and the gastric juice secreted in the following 75 minutes examined for volume, free hydrochloric and total acid secretion. Usually a series of 4 such tests were performed on each animal to determine the average secretory response to histamine. A transthoracic section of the vagus nerves to the stomach was then performed under ether anesthesia with a positive pressure apparatus. The completeness of the vagus section was confirmed in each case by a negative response to insulin hypoglycemia. Following recovery from the vagus section, the animals were tested with histamine exactly as before the operation. The results obtained are indicated in Table I. It is apparent that the volume

* This work has been aided by a grant from the Douglas Smith Foundation for Medical Research, University of Chicago.

¹ Popielski, L., *Pflug. Arch. f. d. ges. Physiol.*, 1920, **178**, 214.

² Keeton, R. W., Luckhardt, A. B., and Koch, F. C., *Am. J. Physiol.*, 1920, **51**, 469.

³ Ivy, A. C., and Javois, A. J., *Am. J. Physiol.*, 1924, **71**, 604.

⁴ Klein, E., *Arch. Surg.*, 1932, **25**, 442.

⁵ Dragstedt, L. R., and Ellis, J. C., *Am. J. Physiol.*, 1930, **93**, 407.

tiary, the tests were performed in a similar manner, except that the gastric content was collected by continuous Wangenstein suction for a period of 60 minutes after the subcutaneous injection of the standard dose of histamine. The postoperative tests were performed from 3 to 6 weeks following the gastric vagotomy when the patients had entirely recovered from the operation. The effect of gastric vagotomy on the secretory response to histamine in the 15 ulcer patients studied at the University of Chicago Clinics is illustrated in Table III. In this series of patients, the vagotomy produced a decrease of 41% in the volume of gastric juice secreted in response to the histamine injection and a decrease of 62% in the total amount of hydrochloric acid produced. The free acid concentration for the 15 tests decreased from an average of 93 clinical units to 67 following the vagus section. Table IV illustrates the results secured

in the 18 patients with peptic ulcer studied at the Illinois State Penitentiary. In these patients, the average volume of gastric juice secreted in response to a standard dose of histamine decreased 56%, and the total acid output 74% after the vagotomy. The average free acid concentration of the histamine stimulated gastric juice averaged 110 clinical units before vagotomy and 48 clinical units after the operation.

Conclusions. 1. The gastric secretory response to a standard dose of histamine in dogs with a totally isolated stomach pouch is markedly reduced by bilateral vagotomy.

2. Atropine similarly reduces the gastric secretory response to histamine in these animals.

3. Gastric vagotomy in patients with peptic ulcer also markedly reduces the gastric secretory response to a standard dose of histamine.

16298 P

Effect of Bile Preparations on Fat Absorption in Bile Fistula Dogs.*

J. R. HEERSMA AND J. H. ANNEGERS. (Introduced by J. S. Gray.)

From the Department of Physiology, Northwestern University Medical School, Chicago, Ill.

That bile is concerned in the absorption of dietary fat has been qualitatively established by the observation of steatorrhea in animals¹ and in man² when bile fails to reach the intestine. The effectiveness of human whole bile in correcting this absorptive defect has been shown in only 2 patients,³ and bile acid preparations have not been tested directly in man or experimental animals. Inconclusive and contradictory results from

studies of blood fat after a fatty meal,⁴ absorption from isolated intestinal loops,^{5,6} and *in vitro* diffusion⁷ or solubilization⁸ constitute the basis for the belief that bile acids are essential for fat absorption, and that

⁴ Crandall, L. A., and Ivy, H. B., *Am. J. Physiol.*, 1940, **129**, 341.

⁵ Plant, O. H., *Am. J. Physiol.*, 1908, **23**, 65; Riegel, C., O'Shea, E. K., and Ravdin, I. S., *Am. J. Physiol.*, 1935, **112**, 669; Doubilet, H., and Reiner, M., *Arch. Int. Med.*, 1937, **59**, 857.

⁶ Virtue, R. W., and Doster-Virtue, *Am. J. Physiol.*, 1942, **135**, 776.

⁷ Verzar, F., and Kuthy, A., *Biochem. Z.*, 1929, **210**, 265; Breusch, F. L., *Biochem. Z.*, 1937, **293**, 280.

⁸ McBain, J. W., Merrill, R. C., and Vinograd, J. R., *Am. Chem. Soc. J.*, 1941, **63**, 670; Mellander, O., and Stenhagen, E., *Acta Physiol. Scand.*, 1942, **4**, 349.

* Supported in part by a grant from G. D. Searle and Company.

¹ Coffey, R. J., Mann, F. C., and Bollman, J. L., *Am. J. Dig. Dis.*, 1940, **7**, 143; Sperry, W. M., *J. Biol. Chem.*, 1930, **85**, 455.

² Hutchinson, H. S., and Fleming, G. B., *Glasgow Med. J.*, 1920, **91**, 65; Thaysen, T. E., *Acta Med. Scand.*, Supplement, 1926, **10**, 384.

³ Shapiro, A., Koster, H., Rittenberg, D., and Schoenheimer, R., *Am. J. Physiol.*, 1936, **117**, 525.

TABLE III.

Effect of Vagotomy on Gastric Secretory Response to Histamine in Patients with Peptic Ulcer.

Patient	Pre-vagotomy		Post-vagotomy		Reduction in vol., %	Reduction in acid output, %
	Vol., cc	Total acid output, m. eq.	Vol., cc	Total acid output, m. eq.		
No. 24	168	10.1	103	5.9	39	41
68	170	10.9	101	2.0	41	81
73	272	28.9	200	11.2	26	61
71	198	19.0	203	12.8	—3	33
74	338	40.6	151	13.6	56	66
17	292	18.8	95	6.5	67	65
22	164	11.6	84	2.7	49	76
23	194	10.3	109	5.2	44	49
24	168	11.0	186	5.5	—1	50
26	353	39.8	131	5.5	63	86
27	192	21.8	130	12.8	32	41
31	143	4.8	68	2.6	53	46
32	63	2.6	45	.8	30	69
34	243	25.6	43	2.5	82	90
35	81	4.0	52	1.1	36	72
Avg	203	17.3	113	6.1	41	62

Note: One mg of histamine phosphate was given subcutaneously in the fasting state and the gastric content removed by continuous suction for 60 minutes.

TABLE IV.

Effect of Vagotomy on Gastric Secretory Response to Histamine in Male Prisoners with Peptic Ulcer.

Patient	Pre-vagotomy		Post-vagotomy		Reduction in vol., %	Reduction in acid output, %
	Vol., cc	Total acid output, m. eq.	Vol., cc	Total acid output, m. eq.		
No. 10	176	22.2	154	16.8	13	24
4	267	28.8	124	11.9	53	59
11	315	42.5	98	3.7	68	91
8	274	30.7	32	3.0	88	90
9	262	31.4	84	4.7	67	85
5	315	27.1	162	15.1	48	44
14	200	20.6	84	3.1	58	85
13	290	33.3	13	.2	95	99
15	210	26.0	180	17.1	14	34
19	232	24.1	28	.4	88	97
22	350	41.6	88	1.8	75	95
21	286	28.6	80	.2	72	99
25	118	8.1	94	6.3	12	22
28	142	13.4	180	9.7	—21	29
26	210	25.6	40	.7	81	97
24	166	15.8	58	1.2	65	92
30	308	40.9	88	1.9	71	95
23	238	25.9	94	.5	60	97
Avg	242	27.0	94	5.5	56	74

ing gastric tube and the Wangenstein suction apparatus. In the morning, one milligram of histamine phosphate was injected subcutaneously and the gastric content aspirated at 10-minute intervals. Similar tests were made from 7 to 10 days after the gastric vagotomy.

The volume of gastric juice secreted during a period of 60 minutes was measured and its free acid concentration determined. From these figures, the total acid output was calculated for each test. In the second group of patients studied at the Illinois State Peniten-

tiary, the tests were performed in a similar manner, except that the gastric content was collected by continuous Wangenstein suction for a period of 60 minutes after the subcutaneous injection of the standard dose of histamine. The postoperative tests were performed from 3 to 6 weeks following the gastric vagotomy when the patients had entirely recovered from the operation. The effect of gastric vagotomy on the secretory response to histamine in the 15 ulcer patients studied at the University of Chicago Clinics is illustrated in Table III. In this series of patients, the vagotomy produced a decrease of 41% in the volume of gastric juice secreted in response to the histamine injection and a decrease of 62% in the total amount of hydrochloric acid produced. The free acid concentration for the 15 tests decreased from an average of 93 clinical units to 67 following the vagus section. Table IV illustrates the results secured

in the 18 patients with peptic ulcer studied at the Illinois State Penitentiary. In these patients, the average volume of gastric juice secreted in response to a standard dose of histamine decreased 56%, and the total acid output 74% after the vagotomy. The average free acid concentration of the histamine stimulated gastric juice averaged 110 clinical units before vagotomy and 48 clinical units after the operation.

Conclusions. 1. The gastric secretory response to a standard dose of histamine in dogs with a totally isolated stomach pouch is markedly reduced by bilateral vagotomy.

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16298 P

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J. R. HEERSMA AND J. H. ANNEGERS. (Introduced by J. S. Gray.)

From the Department of Physiology, Northwestern University Medical School, Chicago, Ill.

That bile is concerned in the absorption of dietary fat has been qualitatively established by the observation of steatorrhea in animals¹ and in man² when bile fails to reach the intestine. The effectiveness of human whole bile in correcting this absorptive defect has been shown in only 2 patients,³ and bile acid preparations have not been tested directly in man or experimental animals. Inconclusive and contradictory results from

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⁵ Plant, O. H., *Am. J. Physiol.*, 1938, **23**, 65; Riegel, C., O'Shea, E. K., and Rardin, I. S., *Am. J. Physiol.*, 1935, **112**, 609; Doubilet, H., and Reiner, M., *Arch. Int. Med.*, 1937, **59**, 857.

⁶ Virnue, R. W., and Dexter-Virnae, *Am. J. Physiol.*, 1942, **135**, 776.

⁷ Verzar, F., and Kathy, A., *Biochem. Z.*, 1923, **210**, 265; Breusch, F. L., *Biochem. Z.*, 1937, **293**, 259.

⁸ McBain, J. W., Merrill, R. C., and Viengrad, J. R., *Am. Chem. Soc. J.*, 1941, **63**, 670; McEnder, O., and Stanhagen, E., *Acta Physiol. Scand.*, 1942, **4**, 349.

* Supported in part by a grant from G. D. Searle and Company.

¹ Coffey, R. J., Mann, F. C., and Bollman, J. L., *Am. J. Dig. Dis.*, 1940, **7**, 143; Sperry, W. M., *J. Biol. Chem.*, 1930, **85**, 455.

² Hutchinson, H. S., and Fleming, G. B., *Glasgow Med. J.*, 1920, **94**, 65; Thaysen, T. E., *Acta Med. Scand.*, Supplement, 1926, **16**, 384.

³ Shapiro, A., Koster, H., Rittenberg, D., and Schoenheimer, R., *Am. J. Physiol.*, 1936, **117**, 525.

TABLE I.
Fecal Fat Excretion in Bile Fistula Dogs.

Regime	No. dogs compared	Means of changes from fecal fat output of 27.4 g/day on no-bile regime, g fat/day	Each regime compared with no-bile, t-ratio
3 g dehydrocholic ac'd	8	+ 1.61	0.71
3 " iron ppt'd ox bile*	7	— 3.20	1.61
3 " desoxycholic acid	5	+ 0.14	0.06
3 " desiccated ox bile†	7	— 1.47	0.72
6 " " " "	6	— 7.50	3.58‡
90 cc " " " "	7	— 14.90	7.60§

* Contains 47% cholic and 48% desoxycholic acids.

† Contains about 50% cholic acid and undetermined desoxycholic acid.

‡ Significant at 5% level.

§ Significant at 1% level.

desoxycholic acid is particularly active. In view of the inadequacy of the indirect, and the lack of direct evidence, studies were undertaken to evaluate the relative effectiveness of various bile acids in restoring normal fat absorption in bile fistula dogs.

Methods. Fecal fat excretion was determined in 9 dogs before and after cholecystonephrostomy, in the latter with and without administration of various bile preparations. The daily diet consisted of 335 g "Pard" with 25 g added lard (36 g total fat). Food was given once daily, and was completely eaten. Each regime consisted of a 7-day period; during the last 5 days feces were pooled and analyzed for total fat.⁹ When given, bile preparations were mixed with the meal (pills, capsules, or fresh bile). Following operation the feces were free of urobilinogen. Neither jaundice nor diarrhea developed, although stools were bulky.

The plan to subject each dog to each regime was not completed because 3 dogs developed duodenal ulcers and 2 dogs did not tolerate 3 g doses of desoxycholic acid. Statistical analyses were made by comparing 2 regimes in the same animal.

Results. The mean fecal fat excretion of the 9 dogs was increased from 3.0 g/day before operation to 27.4 g/day after diversion of bile from the intestine. Three-gram daily doses of bile acids were given in an effort to correct this steatorrhea. This dose is the

maximum that is tolerated for some of the preparations, and is also the 8-hr output of cholic acid by healthy bile fistula dogs when their bile is returned every 8 hours.¹⁰ The failure of iron-precipitated ox-bile (Bilon, Lilly) and desoxycholic acid (Degalol, Ames) to reduce fat excretion was wholly unexpected. Dehydrocholic acid (Ketochol, Searle) was expected to have less effect than the other products tested (Table I). On the basis that the above bile acids may have been altered in their purification, desiccated ox bile and fresh ox bile (kept frozen until use) were administered. Three-gram doses of desiccated ox bile were ineffective; 6-g doses produced a slight but significant reduction in fecal fat. Ninety-cc doses of fresh ox bile, equivalent to 6 g desiccated material, produced a significantly greater, but still incomplete improvement in fat absorption.

Discussion. These results indicate that 3-g doses of certain bile preparations are completely ineffective in replacing the fat-absorptive function of normal whole bile in the dog. It appears that desiccation reduces the ability of fresh ox-bile to promote fat absorption. These results agree with an earlier report⁶ that fresh bile, but not sodium glycocholate or taurocholate, can facilitate the disappearance of sodium oleate from isolated intestinal loops.

No convincing explanation can be offered for these results at present. They are con-

⁹ Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

¹⁰ Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J., *Am. J. Physiol.*, 1941, **131**, 776.

y to general beliefs concerning the role of bile acids in fat absorption, but many speculative possibilities await study. Further direct experimentation is required to determine (a) whether the absorptive defect consequent to bile exclusion can be completely corrected, (b) the identity of the active constituent(s) in bile (c) the effect, if any, of the manner of entry of active material into the intestine, (d) the role, if any, of species

differences, and (e) the mechanism by which fat absorption is facilitated.

Summary. 1. Three-gram doses of certain bile preparations (including desoxycholic acid) proved completely ineffective in reducing the steatorrhea of bile fistula dogs.

2. Ninety-cc doses of fresh ox bile reduced the steatorrhea by 50%, but equivalent doses (6 g) of desiccated ox bile were only half as effective.

16299

Increased Hemolytic Potency of Mouse Mammary Carcinoma Extracts Following Incubation with Tumor Cells.*

LUDWIK GROSS.

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It was recently observed in this laboratory that filtered or centrifugated extracts prepared from spontaneous mouse mammary carcinomas possess the ability to hemolyze mouse erythrocytes *in vitro*.¹

In course of subsequent experiments it was found that tumor extracts that had been separated from the tumor cells by centrifugation, and have been left, in absence of cells, in test tubes, at room temperature, lost practically all hemolytic potency after 5 hours; the decrease in the hemolytic potency of the tumor extracts was even more rapid at 37°C.² This rather striking lack of resistance of the hemolytic factor contained in the tumor extracts, but separated from the tumor cells, to exposure for a few hours to either room, or incubator temperature, was further investigated. One of the first questions requiring clarification was whether the tumor extracts would also lose their hemolytic potency if incubated, at 37°C, in presence of tumor cells.

Accordingly, a series of experiments was performed in which tumor cell suspensions of 20% concentration were freshly prepared from spontaneous mouse (C3H) mammary carcinomas.² Fresh samples of such suspensions were tested for their hemolytic activity.¹ The suspensions were immediately divided into 2 groups of tubes: one was left unchanged as a control, and the other was centrifugated twice at 3,000 t.p.m. for 10 minutes each, to separate the extracts from the tumor cells; following centrifugation, the final supernatant fluid was used. Both, the tumor cell suspensions, and the cell-free extracts, were then placed in an incubator, at 37°C, for 5, and 24 hours. After incubation, samples removed from both groups of tubes were tested for their hemolytic activity by serial titration. Eight different mouse mammary carcinomas were used for the preparation of extracts, and 8 successive series of experiments were performed under apparently identical conditions. The results were as follows:

All 8 fresh tumor extracts were found to possess the usual hemolytic potency¹ when tested before exposure to the incubator temperature. After exposure for 5, or 24 hours, to 37°C, the centrifugated tumor extracts incubated without cells, were found to have lost their hemolytic potency, when tested in

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

¹ Gross, L., *Proc. Soc. Exp. Biol. and Med.*, 1947, 65, 292.

² Gross, L., *J. Immunol.*, 1948, in press.

the usual¹ manner (0.5 cc of an extract of 20% concentration). On the other hand, the same tumor extracts incubated in presence of tumor cells, at 37°C, for either 5 or 24 hours, had not only their hemolytic potency preserved, but either moderately or substantially increased, as compared with the initial titration of fresh extracts. Thus, one of the fresh extracts was found to hemolyze mouse erythrocytes only when tested (0.5 cc) in 20% concentration, *i.e.*, at 1:5. Following incubation in presence of tumor cells at 37°C for 5 hours, the same extract (0.5 cc) hemolyzed readily mouse erythrocytes in dilutions up to 1:40. After an additional incubation with tumor cells at 37°C to a total of 24 hours, this extract hemolyzed mouse erythrocytes in dilutions up to 1:160.

In some instances the increase in hemolytic potency appeared to be slightly more pronounced after 5 than after 24 hours of incubation with tumor cells. In one experiment an extract which had been found to hemolyze mouse erythrocytes, when tested prior to incubation, only at either 1:5, or 1:10, had after 5 hours of incubation at 37°C with tumor cells its hemolytic potency increased to such an extent that serial dilutions up to 1:160 gave positive readings. After an additional incubation with tumor cells to a total of 24 hours, however, the hemolytic potency of the same tumor extract was slightly decreased, giving positive readings in dilutions not exceeding 1:40; thus, it was still higher than that of the initial titration of the fresh tumor extract, but less pronounced than that tested after 5 hours of incubation.

Control Experiments with Normal Cells. It was previously observed^{1,2} that fresh extracts prepared from normal mouse organs, such as liver, spleen, kidneys, muscle, or lungs, had no hemolytic potency on mouse erythrocytes *in vitro* in practically all instances tested,¹ except for extracts made from active mammary glands removed from nursing or pregnant female mice of either a high-tumor, or a low-tumor line.²

In the present study additional experiments were performed in which normal mouse cell suspensions were incubated at 37°C for either 5 or 24 hours, and then tested for hemolytic

potency. Accordingly, normal organs, such as liver, spleen, kidneys, muscle, and lungs, were removed from a total of 12 healthy, adult, virgin female mice of the An (C3H)³ subline, and either individual or pooled cell suspensions of 20% concentration were prepared from each mouse. Fresh samples of the extracts were tested for hemolytic activity.¹ The cell suspensions were incubated at 37°C for either 5 or 24 hours; after this lapse of time, the suspensions were centrifugated, and the supernatant extracts were tested for hemolytic potency in the usual manner.¹ The results were as follows:

Fresh samples of the normal cell extracts had no hemolytic potency.¹ This result was essentially consistent with previous observations.^{1,2}

With the extracts obtained from normal cell suspensions that had been incubated for 5 hours, the following readings were obtained: Of 8 individual liver extracts tested (0.5 cc), 1 was positive in 1:10 dilution. Of 8 pooled (kidney, spleen, muscle, lungs) extracts tested (0.5 cc), 3 were positive in 1:10 dilution. All other extracts, including 4 groups of cell suspensions made from individual organs (lungs, spleen, kidneys, muscle) of 4 females, were negative.

A few tests were made with extracts obtained from cell suspensions that had been incubated for 24 hours. Two of 4 liver extracts, and 2 of 4 pooled extracts were positive (0.5 cc) at 1:10. One of 3 lung extracts tested was positive at 1:5, and 1 of 3 muscle extracts at 1:5, and 1:10. All other extracts, including 3 spleen, and 3 kidney extracts, were negative.

Summary. If separated from the tumor cells by centrifugation, the mouse mammary

† This was true under routine conditions of the test, *i.e.*, incubation of the extracts with mouse erythrocytes at 37°C for 2½ to 3 hours.¹ It was found, however, that normal mouse organs may occasionally show a "delayed" hemolytic potency; thus, when extracts prepared from normal mouse cells were mixed with mouse erythrocytes, and incubated first at 37°C for 5 hours and then at 7°C (refrigerator) for additional 15 hours, a slight hemolysis resulted in some instances.

³ Gross, L., *J. Immunol.*, 1947, **55**, 297.

carcinoma extracts lose their hemolytic potency, when incubated at 37°C, after 5 hours. On the other hand, the hemolytic potency of the tumor extracts is either moderately, or substantially increased following incubation in presence of tumor cells, at 37°C, for either 5 or 24 hours.

Fresh extracts prepared from normal mouse cells have in most instances no hemolytic

potency, except for those made from active mouse mammary gland.² If the normal mouse cell suspensions (liver, lungs, muscle, or pooled organs), however, are incubated at 37°C for 5, or better for 24 hours, some of them may show a slight hemolytic potency, though to a lesser degree than that observed in the case of pre-incubated tumor cell suspensions.

16300

Reconstitution of the Dermal Barrier to Fluid Diffusion Following Administration of Hyaluronidase.*

OSCAR HECHTER. (Introduced by David Rapport.)

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It is now generally accepted that hyaluronidase owes its spreading activity to the action of the enzyme upon a viscous hyaluronic acid gel in connective tissues^{1,2} which is one of the components of the tissue barrier to interstitial fluid diffusion.³ The effect of hyaluronidase in facilitating the spreading in skin remains evident in the locally treated area for as long as 24 hours after administration of the enzyme, and thereafter is absent.⁴ This demonstrates that after a certain latent period, the organism replaces the component of the skin barrier removed by the hyaluronidase.

It appeared likely that a quantitative study of the rate at which the dermal barrier to spreading of fluids was restored, after administration of hyaluronidase, might provide information concerning the formation of new dermal hyaluronic acid *in vivo*. Accordingly, such a study was undertaken in adult humans.

The hyaluronidase used was a purified bovine testis preparation (Schering) which assayed 20 turbidity-reducing units (TRU) per mg as evaluated by the method of Kass and Seastone.⁵ The enzyme was used directly

after dissolution in 0.85% sodium chloride. Three men and 3 women received intradermal injections of 0.20 cc of enzyme solution containing 20, 2, 0.2, 0.02, 0.002, and 0.0 TRU per cc into separate areas of the 2 arms. After the injection of the enzyme solutions, the local areas were marked with tincture of merthiolate. The time of wheal disappearance following intradermal injection served as a measure of hyaluronidase spreading activity. The local area was examined continuously for 10 minutes after the injections, then at 5-minute intervals until the wheal has disappeared, *i.e.*, until the injected local area appeared completely flat as viewed from a 90° angle. At 24, and then again at 48, hours after the initial injections of enzyme 0.20 cc of an 0.85% solution of NaCl was injected into each previously treated area, and the time of wheal disappearance determined.

Table I illustrates the interrelationships between the time required for wheal disappearance and the concentration of enzyme injected. On the assumption that the time of wheal disappearance is a measure of the barrier efficiency of the dermal hyaluronic acid gel associated with the tissue barrier to

* Aided by a grant from the G. D. Searle & Co.

¹ Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

² Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

³ Hechter, O., *Fed. Proc.*, 1947, **6**, 126.

⁴ McClean, D., *J. Path. and Bact.*, 1930, **33**, 1045.

⁵ Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1941, **70**, 319.

TABLE I.

The Wheal Disappearance Time of Intradermally Administered Saline, Injected into Areas Treated with Hyaluronidase.

No. of subjects	Amt of hyaluronidase,* T.R.U.	Time of saline injection after hyaluronidase				
		0 hr	24 hr		48 hr	
		W.D.† min.	W.D. min.	B‡ %	W.D.† min.	B‡ %
6	4	0.20 ± 0.06‡	6.50 ± 1.8	11	62.5 ± 6.6	100
6	4 × 10 ⁻¹	0.78 ± 0.17	13.7 ± 2.8	23	61.7 ± 7.2	100
6	4 × 10 ⁻²	3.0 ± 0.70	24.7 ± 4.3	41	61.7 ± 8.5	100
6	4 × 10 ⁻³	6.1 ± 1.2	36.7 ± 4.7	61	67.5 ± 5.9	100
6	4 × 10 ⁻⁴	15.0 ± 5.6	46.7 ± 6.2	78	63.3 ± 6.6	100
6	0	65.0 ± 8.4	58.0 ± 9.0	—	61.1 ± 7.8	—

* Intradermal administration in a constant volume of 0.20 cc.

† W.D. is the mean time of wheal disappearance.

‡ Is the standard error of the mean.

§ B is the percentage of the normal effective barrier present after enzyme administration assuming (a) a linear relationship between effective barrier and spreading and (b) that a normal barrier has a wheal disappearance time of 60 minutes.

diffusion, the percentage restoration of dermal hyaluronic acid 24 and 48 hours after the administration of enzyme has been calculated and these values recorded in Table I.

Twenty-four hours after the administration of hyaluronidase in amounts ranging from $4 \times 4 \times 10^{-4}$ TRU (in a volume of 0.2 cc), the dermal barrier of the local area of injection exhibited a permeability which was directly related to the dose of the enzyme; after 48 hours, the permeability of the barrier in all treated areas was decreased to normal.

In skin, purified testis hyaluronidase appears to act specifically upon hyaluronic acid² and does not affect other mucopolysaccharides, such as chondroitin sulfate, which is present in a concentration equivalent to that of hyaluronate.² This does not hold for other tissues, purified testis hyaluronidase having been reported to hydrolyze the monosulfuric acid ester of hyaluronic acid obtained from cornea⁶ and the chondroitin sulfate of hyaline cartilage.^{7,8} We have found that injection of hyaluronidase

into areas which have "recovered" from a previous dose of enzyme produces its usual spreading reaction, thus strongly suggesting that new dermal hyaluronate, and not another mucopolysaccharide, insensitive to hyaluronidase, is involved in the reconstitution of the barrier.

Summary. The reconstitution of the dermal barrier removed by intradermal injection of various doses of hyaluronidase has been studied 24 and 48 hours after enzyme injection in adult humans. At 24 hours the restoration of the barrier is incomplete and inversely related to the dosage of enzyme; at 48 hours the barrier is completely restored in all enzyme-treated areas.

⁶ Meyer, K., and Chaffee, E., *Am. J. Ophthalm.*, 1940, **23**, 1320.

⁷ Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

⁸ Madinaveitia, J., and Stacey, M., *Biochem. J.*, 1941, **38**, 413.

Hepatic Fibrosis in the Persistently Non-Fatty Liver of the Hypophysectomized Dog.

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The frequent association of hepatic lipoidosis and necrosis with cirrhosis in man and experimental animals has led to the view that fatty and other forms of liver-cell degeneration induce cirrhosis. Where fatty changes did not precede hepatic fibrosis, hemorrhagic or some other form of hepatocellular necrosis has usually been considered to be the precipitating lesion.

That some factor or factors other than liver-cell damage may possibly be responsible for the initiation and progress of hepatic fibrosis is brought out in the present investigation. Fibrosis was found in livers of hypophysectomized dogs in which the fatty-acid content never exceeded 4%, *i.e.*, normal concentrations, and in which evidence of extensive liver-cell destruction was not detected histologically.

Experimental Procedures. For the first 3-4 weeks after their arrival at the laboratory the dogs were fed a diet high in protein and adequate in all respects. Each dog received twice daily, at 8:00 a.m. and at 4:00 p.m., 15 g of lean meat per kilo, 10 g of sucrose, 4 g of bone ash, and 1 g of Cowgill's salt mixture.¹ Vitamin supplements were provided by the addition of 3 cc Sardilene[†] and 5 cc of Galen B.[†] After the dogs had received this diet for 2-3 weeks, they were hypophysectomized by a method previously described.³ In the pro-

cedure used in this study no cautery was applied to the base of the brain. In most cases the appetites of the dogs were excellent after hypophysectomy.

At the end of the periods of observation recorded in Table I, the whole livers of the dogs were excised while they were under nembutal anesthesia. Several sections of each liver were removed for histological study, and the rest was ground and sampled for lipid analysis as described in a previous paper.⁴

Serial sections were made of the base of the brain of each dog and every fifth section carefully examined. No cells of the anterior, posterior, or intermediate lobes of the pituitary gland were found in the dogs recorded in this study; nor was there any evidence of damage to the hypothalamic region.

Results. The chemical and histological findings in the livers of these animals have been summarized in Table I. In an earlier study the fatty-acid contents of dogs' livers were examined at intervals of 2-4 months after hypophysectomy;⁵ no deviation from the normal was found at these time-intervals. The results recorded in Table I leave no doubt that hypophysectomy *per se* does not influence the fat content of the liver, even 32 months after it has been performed.

Histologically, the liver cells did not show any evidence of fatty change or necrosis. The only abnormal findings in the liver cells were firstly an unusual distinctness of the cell walls with clumping of the chondriosomes (plant-like cells), and secondly an eosino-

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¹ Cowgill, G. R., *J. Biol. Chem.*, 1923, **50**, 725.

[†] Each cubic centimeter of Sardilene contained not less than 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A. The vitamin content of Galen B has been recorded elsewhere.²

² Montgomery, M. L., Entenman, C., Chaikoff, I. L., and Nelson, C., *J. Biol. Chem.*, 1941, **137**, 693.

³ Dandy, W. E., and Reichert, F. L., *Bull. Johns Hopkins Hosp.*, 1925, **37**, 1.

⁴ Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **100**, 267.

⁵ Chaikoff, I. L., Gibbs, G. E., Holtom, G. F., and Reichert, F. L., *Am. J. Physiol.*, 1936, **110**, 543.

TABLE I. Pathological Findings and Fatty Acid Content of Livers of Hypophysectomized Dogs.

Dog	Body wt		Sacrificed after hypophy- sectomy, mo.	Liver findings				Total fatty acids, %
	Initial, kg	Max., kg		Pathological		Eosinophilic hyalinization		
				Fibrosis*	Cirrhosis†		Plant-like cells	
H25	7.2	10.0	24	3+	early	2+	220	2.2
H26	7.6	8.6	24	0	0	2+	170	1.9
H27	7.2	8.1	24	patchy slight	0	2+	152	4.9
H28	6.2	8.2	24	4+	+	2+	176	2.7
H29	6.4	10.6	24	3+	+	2+	196	3.9
H32	4.1	8.3	32	0	0	3+	265	3.8
H33	5.5	6.7	32	0	0	3+	212	3.2

* *Hepatic fibrosis* or precirrhosis indicate the presence of abnormal amounts of fibrous tissue in the liver without architectural distortion of the lobular pattern.

† *Cirrhosis* indicates the supervention of architectural distortion in association with hepatic fibrosis.

‡ *Histological grading* of "plant-like" cells and eosinophilic hyalinization:

+ = Scattered foci

2+ = About 1/4 of lobule affected.

3+ = About 1/2 of lobule affected.

Assessment of degree of fibrosis:

+ = Present and in scattered foci.

3+ = About 1/2 of lobules affected.

4+ = About 3/4 of lobules affected.

philic hyalinization of the liver cells. Both these changes were widespread in the livers of *all* the dogs and apparently occurred independently of the incidence of fibrosis. While the eosinophilic hyalinization occurred in all the livers, this reaction was most prominent around the non-fibrosed portal tracts (Fig. 1). Apart from these changes in the liver cells, the majority of livers appeared relatively avascular by virtue of the fact that most of sinusoids were closed and did not contain blood. This occlusion of the sinusoids and the close apposition of the individual liver cells to one another gave the liver a highly cellular appearance (Fig. 1).

The fibrotic reaction to be described below was detected, in varying severity, in 4 of the 7 hypophysectomized dogs. This connective-tissue reaction was most unusual in its form, location, and distribution in the affected livers. In the first place not all the lobes of the same liver were affected by this reaction and, had we not had sections from several parts of each liver, we would clearly have missed the change in at least 2 of the dogs which manifested this lesion. It is possible that the patchy distribution of this lesion was due, in several of our dogs, to the fact that it had begun only a short time before death. It is probable that, had the dogs been maintained for longer periods of time, the fibrosis would have become both more extensive and more severe.

When present, the fibrosis which we detected was most frequent and most severe in and around the portal tracts, although by no means confined to these regions (Fig. 2). The fibrosis took two forms, which possibly represented different stages in the evolution of the lesion. In its most striking form it appeared as a highly cellular connective-tissue proliferation (Fig. 2, 3, and 4). This cellularity was due primarily to the large numbers of fibroblast and fibrocyte nuclei. The portal tracts, both large and small, were made very prominent by the accumulation around them of round cells and numerous closely packed, spindle-shaped, young-fibrous-tissue nuclei (Fig. 2 and 3). Often, when sections were cut obliquely through what, by location, seemed to be portal tracts, sheets of this highly

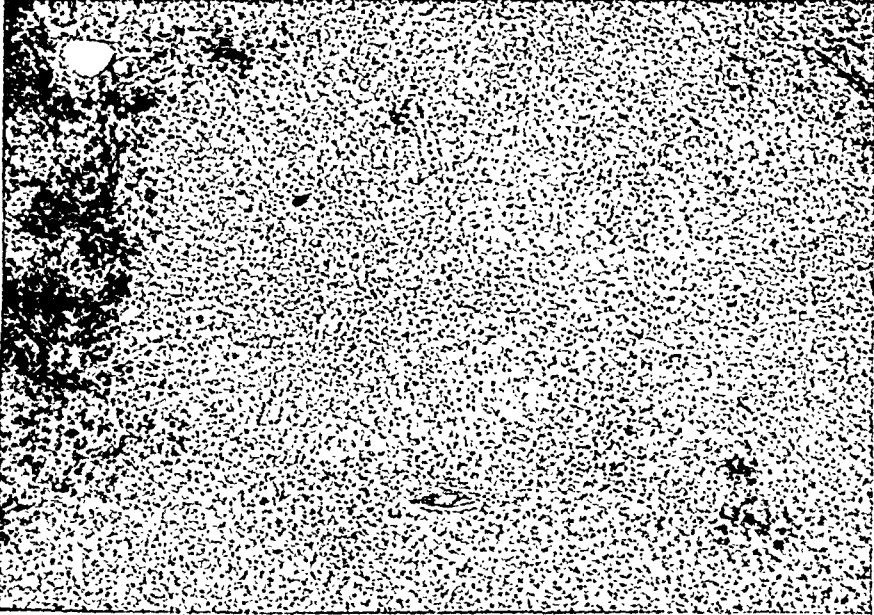


FIG. 1.

(H32) The periportal localization of the eosinophilic hyalinization with nuclear hyperchromasia of the liver cells is clearly shown. Note also "plant-like" cells in the rest of the lobule and the avascularity of the liver. Note also the absence of fatty change. Hematoxylin and Eosin $\times 45$.

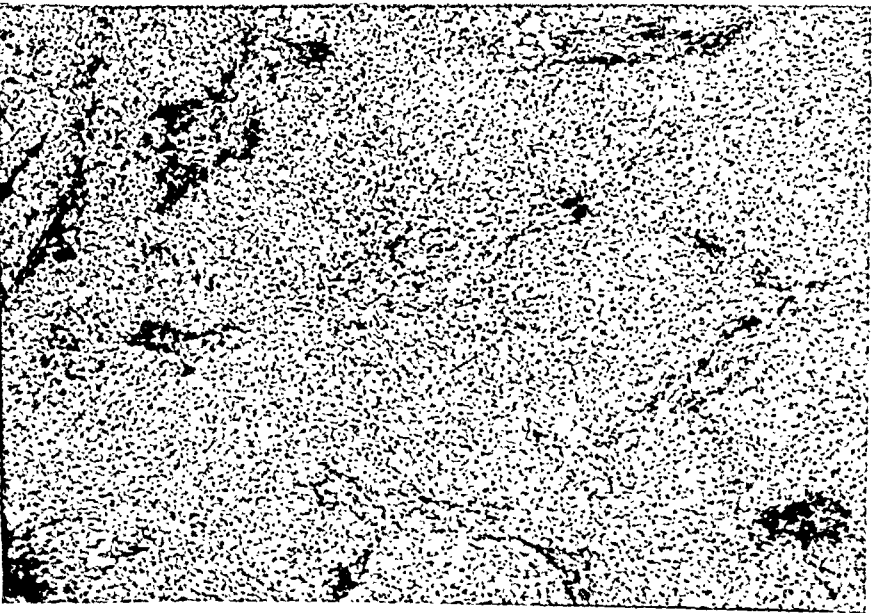


FIG. 2.

(H28) The cellular periportal fibrosis and the less highly cellular reaction in the central portion of the lobule are typical of the changes seen in these livers. Hematoxylin and Eosin $\times 70$.

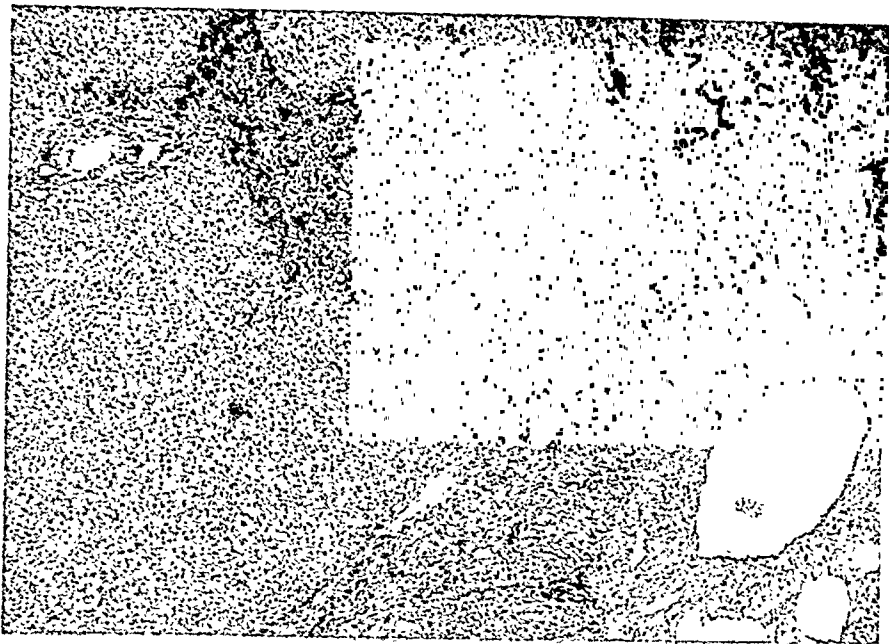
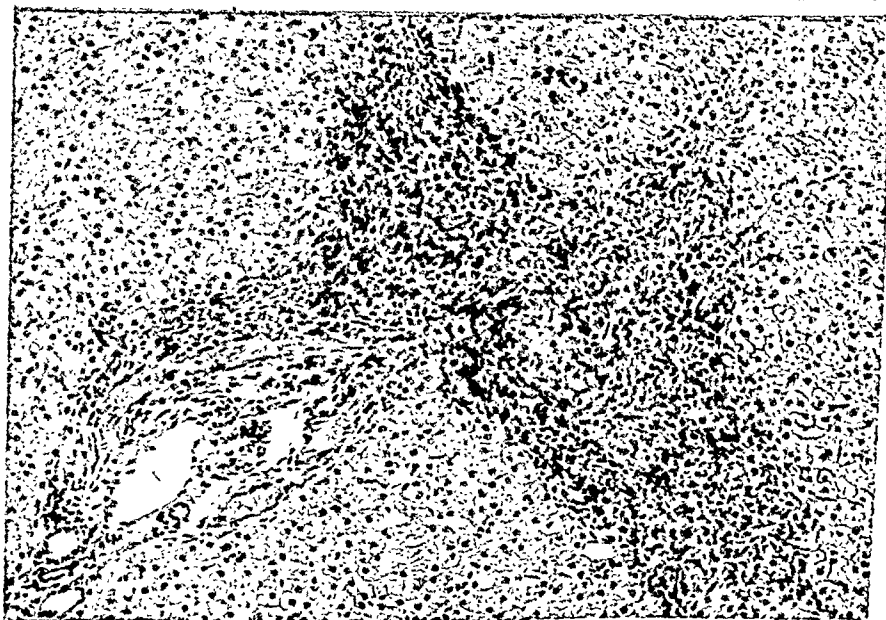


FIG. 3.

(H29) The portal tract towards the top left corner of the picture is made prominent by the marked accumulation of cells. Scattered bands of fibrous tissue can also be detected in this picture. Hematoxylin and Eosin $\times 64$.

FIG. 4.

(H29) Details of the nature of the cells infiltrating the portal tract depicted at the top left corner of Fig. 3. In addition to round cells there are large numbers of spindle-shaped fibrocytic nuclei. Hematoxylin and Eosin $\times 152$.



cellular connective tissue seemed to be stretched across the liver lobules. However, in other areas these sheets of young connective tissue seemed to be related to radicles of the hepatic veins or occurred in the middle of apparently intact liver-cell lobules (Fig. 2). Such masses of young connective tissue occasionally joined together, thus outlining 2 sides of a lobule, or, when more extensive, it obliterated a liver lobule. This latter reaction when present was most common in the subcapsular regions, although in the more severely affected livers such obliteration of liver tissue was more widespread.

The great cellularity of this connective tissue was very similar to that detected by Graef *et al.*⁶ in the fatty livers of their hypophysectomized dogs (*cf.* their Fig. 5 and 6 with our Fig. 2 and 3). However, in many of the fibrosing areas the cellularity was made less prominent by the fact that masses of dense, highly eosinophilic collagen fibers had been deposited among the still numerous fibroblast and fibrocyte nuclei (Fig. 2 and 3).

Associated with this fibrous tissue were numerous histiocytic cells containing very dark-brown coarse pigment granules. Similar pigmented histiocytes have been encountered in smaller numbers in the livers of normal dogs, but such pigmented cells were not detected in the non-fibrotic livers of hypophysectomized dogs.

Discussion. The relation of liver-cell damage to the onset of cirrhosis has been much discussed in recent years. Although the connective-tissue changes in response to hypophysectomy are not so pronounced as those encountered in livers in which extensive fatty infiltration or necrosis has been induced, the present findings leave no doubt that the stimulus to connective-tissue proliferation in the liver need not be hepatocellular degeneration, either fatty or necrotic. The greatest amount of fibrous-tissue proliferation occurred in the livers of dogs H25, H28, and H29; in these, fatty change or degeneration of the liver cells was not detected.

The mechanism by which hypophysectomy brings about fibrous-tissue proliferation in the

liver is not clear at present. Graef *et al.* stated that "it appears reasonable to conclude that hypophysectomy itself . . . had little if anything to do with the hepatic lesions encountered in these dogs. The hypothalamic lesions disclosed by histological examination seemed to have a pivotal role in the hepatic changes." Careful histological studies of the hypothalamic regions in *our* animals, using the serial-section technic mentioned above, failed to reveal any evidence of damage to this important center. This fact, together with the absence of fat from the livers of our hypophysectomized dogs, indicates that the pathogenesis and possibly the etiology of the hepatic fibrosis in our animals differed somewhat from those described by Graef *et al.*

The protein content of the diets of our animals was high, and consequently low-protein *intake* could not be blamed as a factor in the etiology of the hepatic fibrosis in them. However, some interference in the absorption process has been shown to follow excision of the pituitary gland.^{7,8} Hence despite the ingestion of a high-protein diet by our dogs, it is conceivable that hypophysectomy induced some disturbance in the digestion or absorption of certain dietary substances which play a critical role in maintaining a normal liver. The possibility that hypophysectomy promotes an increased need for such substances is, of course, not ruled out.

Summary. 1. Fibrous-tissue proliferation is shown to occur in the livers of hypophysectomized dogs. Its development was not preceded by an increase in the fat content of the liver.

2. Hypothalamic damage as a cause of the fibrous-tissue proliferation was ruled out.

3. The development of hepatic fibrosis in these dogs fed a diet adequate in all respects and rich in proteins suggests that hypophysectomy induces either (a) a derangement in digestion and/or absorption, or (b) an increased need for certain dietary substances essential for the maintenance of a normal liver.

⁷ Russell, J. A., *Am. J. Physiol.*, 1938, **121**, 755.

⁶ Graef, I., Negrin, J., and Page, I. H., *Am. J. Path.*, 1944, **20**, 823

⁸ Althausen, T. L., *Essays in Biology in Honor of H. M. Evans*, University of California Press, Berkeley, 1943, p. 13.

A Quantitative Study of the Effect of Vagotomy on Gastric Secretion in the Dog.*

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Lim, Ivy, and McCarthy¹ described the use of a pouch of the entire stomach in studying the physiology of gastric secretion in the dog. This preparation is sometimes called a Fremont pouch, since Fremont² described this procedure in 1895.

In the total gastric pouch used by Lim, Ivy, and McCarthy, the vagus nerves to the stomach were divided when the cardia was transected. These dogs secreted 200 to 300 cc of gastric juice with a relatively low free acidity. Dragstedt and Ellis^{3,4} described the total gastric pouch prepared with the vagus nerves intact. Unlike the preparation used by Lim, Ivy, and McCarthy, such an animal secretes 600 to 2,000 cc of highly acid gastric juice daily, and will succumb to hypochloremia and alkalosis in 3 to 10 days unless given daily intravenous infusions of Ringer's solution. These animals develop large penetrating peptic ulcers in the gastric pouch,⁵ and frequently die of perforation or hemorrhage.

The present study was undertaken to determine quantitatively the effect of vagotomy on hydrochloric acid and pepsin secretion in the total pouch dog, and to determine the effect of vagotomy on secretion in response to food and other stimuli. A total of 12 dogs survived surgery. Vagotomy was performed in 9 animals by the transthoracic approach.

* This work was aided by grants from the Kenneth Smith Fund for Medical Research and the Douglas Smith Foundation for Medical Research.

¹ Lim, R. K. S., Ivy, A. C., and McCarthy, J. E., *Quart. J. Exp. Physiol.*, 1925, **15**, 13.

² Fremont, *Bull. de l'Acad. de Med.*, 1895, **34**, 509.

³ Dragstedt, L. R., and Ellis, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1929, **26**, 305.

⁴ Dragstedt, L. R., and Ellis, J. C., *Am. J. Physiol.*, 1930, **93**, 407.

⁵ Matthews, W. B., and Dragstedt, L. R., *Surg., Gynec., and Obst.*, 1932, **55**, 265.

With the animals on a standard diet, the 24-hour gastric secretion was collected, titrated for free acidity, and the peptic power determined by a method described by LeVeen.⁶

In each animal, the integrity of the vagus nerves to the gastric pouch was established by the use of the intravenous insulin test, as described by Ihre⁷ and Jemerin, Hollander, and Weinstein.⁸ In one dog, the test was negative, and secretion remained low in volume and acidity. The response to histamine was determined by injecting one milligram subcutaneously and collecting the gastric juice over a 75-minute period. The response to food-taking was measured by feeding the fasting animal a standard meal of meat over a 15- to 30-minute period, and by collecting fractional samples of gastric juice for 12 to 16 hours. The same testing procedures were carried out following vagotomy.

Vagotomy produced a decrease in the volume and free acidity of the 24-hour gastric secretion in all of the 9 animals which were vagotomized. The decrease in volume varied from 37 to 79%, averaging 56%. The decrease in free acidity varied from 14 to 82%, averaging 54%. The decrease in milliequivalents of free hydrochloric acid secreted varied from 47 to 92%, the average reduction in hydrochloric acid output being 77%.

In 7 of 8 animals, vagotomy produced a reduction in the peptic power of the 24-hour secretion. This reduction varied from 11 to 72%, averaging 34%. In one animal, the peptic power increased by 47%.

⁶ LeVeen, H. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 254.

⁷ Ihre, B. J. E., *Acta Med. Scandinav. Suppl.*, 1938, **95**.

⁸ Jemerin, E. E., Hollander, F., and Weinstein, V. A., *Gastroenterology*, 1943, **1**, 500.

Vagotomy produced a marked decrease in secretion to histamine stimulation:

In 3 of 4 animals tested, secretion was high in volume and acidity in the fasting state. Within 15 minutes after feeding, there was a pronounced augmentation in secretion, followed in 15 to 30 minutes by a profound inhibition in volume, free acidity, and peptic power. After 3 to 7 hours, secretion returned to the fasting level. Following vagotomy, there was no augmentation with feeding, and the period of inhibition appeared as before.

The ineffectiveness of partial vagotomy was well demonstrated in 2 animals. In one the left, in the other the right vagus nerve was crushed in the cervical region. The secretory response to insulin hypoglycemia was unaffected, and there was no diminution in the 24-hour secretion either in volume or acidity.

Three dogs died of peptic ulcer. Two died of perforation 4 and 7 days after operation; the third died of hemorrhage 5 days after vagotomy.

Conclusions. 1. The totally isolated stom-

ach with intact blood and nerve supply secretes large amounts of gastric juice even in the absence of food-taking.

2. On the ingestion of food there occurs an immediate augmentation of secretion followed by a period of inhibition lasting 3 to 7 hours and then a period of profuse secretion.

3. Section of the vagus nerves above the diaphragm reduces the secretion of gastric juice in the isolated stomach by an average of 56% and the output of hydrochloric acid by 77%. Nervous factors are thus more important than other mechanisms in determining gastric secretion in these animals.

4. Chronic progressive peptic ulcers occur frequently in these isolated stomachs and cause death by hemorrhage or perforation. They rarely develop in such preparations that have been denervated, and following vagotomy, they tend to heal.

5. Partial vagotomy has little or no effect on gastric secretion.

6. After complete vagotomy, the secretory response to a standard dose of histamine is markedly reduced.

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Scarring and Precirrhosis of the Liver in Chronic Phosphorus Poisoning of Guinea Pigs.

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In a previous report,¹ we described the fibrous tissue in dietary cirrhosis of rats and carbon tetrachloride cirrhosis of rats and guinea pigs as forming primarily about the hepatic veins. It was suggested that at least in certain experimental cirrhoses the proliferation and condensation of fibrous tissue occur in the same part of the liver lobule as the parenchymal damage. It seemed desirable to test this concept further by repeatedly injuring the peripheral (portal) part of the

liver lobules to determine whether or not fibrous tissue would form about and connect the portal areas.

White phosphorus was selected for use in this study since it has been stated that this substance produces periportal fatty degeneration and necrosis of the liver, and since Mallory,² among others, has produced cirrhosis in rabbits with phosphorus.

We were not successful in producing frank liver cirrhosis in the animal used (guinea pig). However, other striking extensive

¹ Ashburn, L. L., Endicott, K. M., Daft, F. S., and Lillie, R. D., *Am. J. Path.*, 1947, **23**, 159.

² Mallory, F. B., *Am. J. Path.*, 1933, **9**, 557.

asymmetrical lesions were observed; a description of these changes is the main purpose of this paper.

Experimental Methods. Fifty-one guinea pigs weighing between 300 and 500 g were divided into 2 groups and fed a diet of rabbit pellets and leafy vegetables. The animals in one group received 0.75 mg/kg of phosphorus 4 days each week, while those in the second group were given 1.5 mg/kg twice weekly. The phosphorus was made up as a 0.1% solution in olive oil. This solution was administered orally by the use of a 1 cc tuberculin syringe and a 15-gauge needle one inch long; the end was made slightly bulbous by a small amount of solder. The guinea pigs were held in a vertical position and the proper dose was expelled slowly into the back of the throat. The dose of phosphorus used in the study was, for certain periods, above the tolerance limit of some animals; so, based on the appearance of the animal and the weight curve, it was sometimes necessary to reduce or omit single doses. Occasionally 2 or more successive doses were reduced or omitted to obtain a more satisfactory weight curve. The experiment was continued for a period of 35 weeks. In order to study the anticipated changes as they progressed, from 2 to 4 animals were killed at irregular intervals beginning in the first week.

At autopsy most of the livers were injected as previously described¹ with a charcoal gelatin mass to mark the hepatic or portal veins in the microscopic section. Livers were fixed either in 10% formalin or Helly's fluid, paraffin sectioned, and stained by the Van Gieson technic and with azure eosinate. Some were also stained with hematoxylin and eosin, and by Foot's modification of Rio Hortega's silver method for demonstrating reticulum. In addition frozen sections were stained with oil red O.

Results. An analysis of the results showed no difference in incidence or type of lesions found in the livers of the animals given phosphorus four days a week and those given this substance twice a week; hence all animals will be considered as one group.

Gross Findings. The first gross lesions were seen in an animal killed 9 weeks after

the beginning of the experiment. This liver showed, on its ventral aspect, dark reddish brown areas at the hilar portion of most lobes with extension for short distances toward the free borders. The lesions were sharply margined, depressed, and suggested a loss of parenchymal substance. As the time on the experimental regimen increased, lesions were seen with greater frequency and extensiveness, and involving a greater number of lobes. These lesions varied considerably in size and shape. They were triangular, oval, stellate, or linear, and when isolated they measured up to 2 cm in their greatest dimension. Although they were commonly seen in the hilar region, they were by no means limited to this area. Various lobes of the same or different livers showed variously deep notches along their margins, extreme atrophy of the proximal segment, punched-out areas on dorsal or ventral surfaces, or were incompletely traversed by fissures. When these changes occurred in a single lobe, there was great distortion. Occasionally atrophy and shrinkage of one or more lobes were extreme. When the accessory lobes were involved in this fashion, the gall bladder bed was thus lost and this organ was found in abnormal locations. In one liver the left main and right and left accessory lobes were shrunken to the point where their combined mass measured only 2 x 1 x 0.2 cm (Fig. 1E). The right main and caudate lobes of this liver showed no focal lesions but were considerably enlarged. Hypertrophy was generally present in uninvolved lobes when others showed marked atrophy. The distal third of lobes was rarely involved. This portion was sometimes hypertrophied and connected with the remainder of the liver by a shrunken band of tissue representing the proximal two-thirds of the lobe. Nodular hyperplasia was not seen.

Examination of the cut surface occasionally showed small lesions which did not reach the surface. The externally evident lesions penetrated the liver for varying depths, usually less than half of the thickness of the lobe. At times the entire thickness of the lobe was involved.

The incidence of involvement of the various

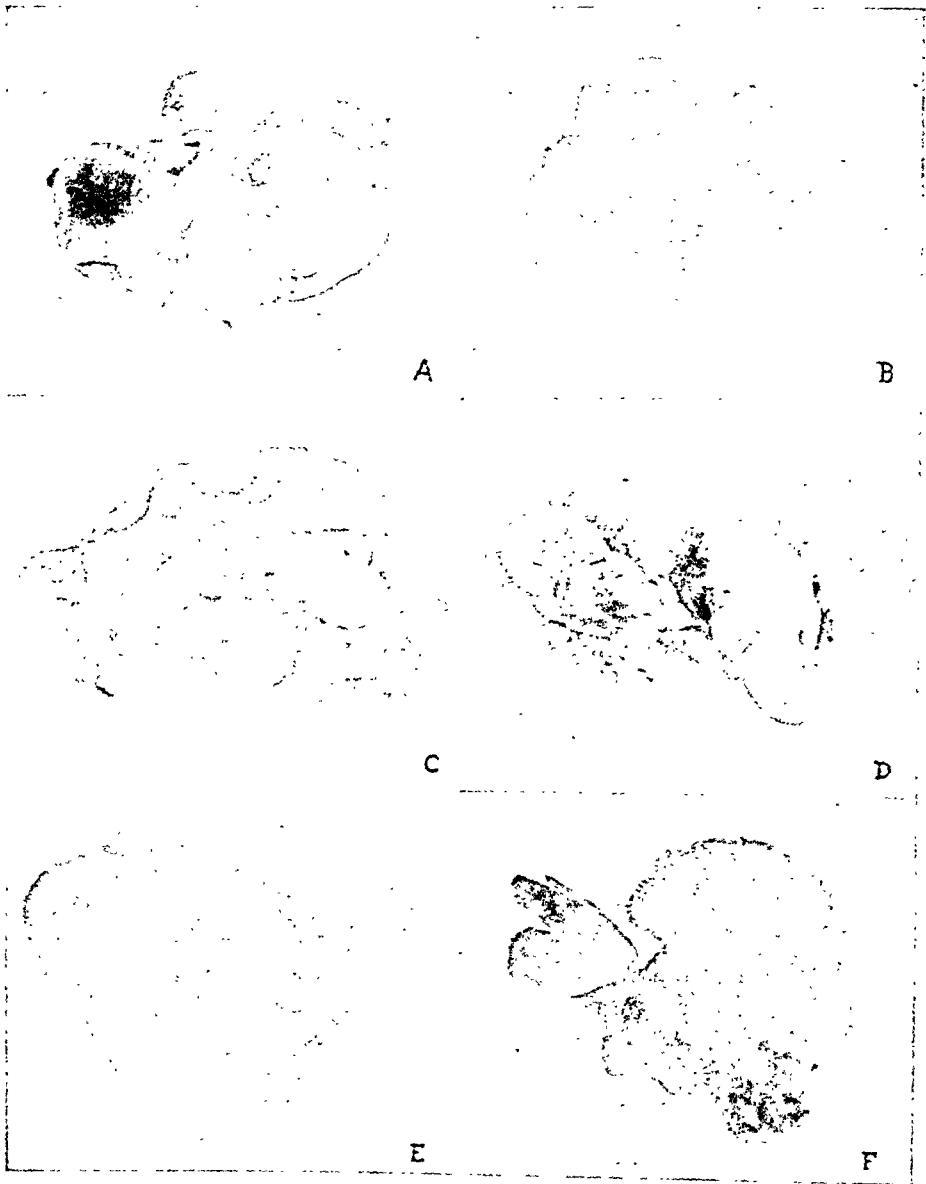


FIG. 1.

Gross photographs of guinea pig livers showing various degrees of deformity following chronic phosphorus poisoning.

- A. Diaphragmatic surface—shows defects along margins of lobes.
- B. Diaphragmatic surface—similar to A, but more advanced changes.
- C. Inferior surface—numerous deep notches and fissures give appearance of nodular hypertrophy. The gall bladder has become detached from its bed (enlarged notch at top) and lies in a horizontal position.
- D. Diaphragmatic surface. There is marked atrophy of the right accessory lobe (dark area in center of photograph), fore-shortening of right main, and atrophy of proximal portion of caudate lobe. The atrophic portion of caudate is largely covered by the right main lobe.
- E. Inferior surface. The small mass of tissue to the right of, and below the gall bladder is all that remains of the left main and left and right accessory lobes.
- F. Diaphragmatic surface. The large mass of tissue is the left main lobe. All other lobes are atrophic.



FIG. 2.
Photomicrographs of lesions of guinea pig livers in chronic phosphorus poisoning. Three of the livers were injected with a carbon-gelatin mass.

A. Histologically early lesion showing almost complete disappearance of parenchymal cells,

blood in some sinusoids and no appreciable fibrosis. Hepatic veins were injected. Hematoxylin and eosin stain, $\times 120$.

B. Similar to A but from a different liver. Area was selected to show a few small groups of remaining liver cells and more blood in the non-collapsed sinusoids. Van Gieson stain, $\times 160$.

C. The liver below the hepatic artery is normal. Superficial to it there is subtotal disappearance of liver cells with collapse of sinusoids. Hematoxylin and eosin stain, $\times 25$.

D. Late lesion. Liver cells are replaced by dense fibrous tissue. Note sections of the tortuous hepatic artery. Hepatic veins were injected. Van Gieson stain, $\times 100$.

E. Thin partially fibrous trabeculae connect many portal areas. Prominent hepatic veins are seen within some of the enclosed lobules. Van Gieson stain, $\times 30$.

F. Carbon-gelatin mass was injected in the portal vein. Four portal and three hepatic veins are shown. There is marked reduction in size of lobules but no increase in connective tissue. Azure eosinate stain, $\times 90$.

lobes was irregular, with no particular lobe showing outstanding susceptibility to injury. The right accessory lobe was involved in 22 livers, the left accessory in 19, the right main in 19, the left main in 15, and the caudate in 10. Although the caudate showed the lowest incidence of gross lesions, it revealed the greatest relative incidence of extensive damage. Of the 10 instances of caudate involvement, the lesions were considered extensive in five. This contrasts with the findings in the left main lobe in which only 3 of 15 showed extensive lesions. The other lobes fell between these extremes.

Microscopic Findings. The lesions noted grossly varied in microscopic appearance. This variation was considered as a function of age of the lesions and this interpretation is used to facilitate the description of microscopic appearance. The early lesions were of variable size and showed from moderate to subtotal disappearance of liver cells (Fig. 2, A and B). Sinusoids in most lesions were generally collapsed. In some they were open and blood-filled. In areas of collapse, there was both an apparent and an actual increase in cellularity since large mononuclear cells and occasionally lymphocytes were present in variable numbers. The liver cells remaining in such areas occasionally showed hydropic, fatty or other degenerative changes. Hyperplasia of the remaining cells was not seen. In these early lesions connective tissue fibers were not numerous and reticulum fibers predominated.

In most lesions bile ducts appeared to be present in greater number than could be accounted for by a collapse of lobules. This belief was supported by the presence of biliary epithelium arranged in cords without lumens and by the presence of atypical cords. Bile

duct proliferation was never more than moderate and often was only of slight degree.

As the lesions became older they showed an increased amount of fibrous tissue, a decrease in cellularity, somewhat fewer bile ducts and fewer liver cells. What appeared to be the end stage of these lesions was seen in a few livers after the twenty-third week. They showed moderate to marked fibrosis, small to moderate numbers of spindle cells, scattered normal bile ducts and, in some cases, numerous sections of the convoluted or undulating hepatic arteries (Fig. 2D). Occasionally a lesion was seen in which only patchy areas showed fibrosis.

Extensive necrosis was seen in only one animal. This occurred in a liver in which the portal areas were markedly widened due to disappearance of adjacent parenchymal cells, cellular infiltration and proliferation of bile ducts. The majority of the liver cells between such areas were in various stages of karyorrhectic necrosis. Some lobules in this area of involvement showed no liver cells; only blood-filled sinusoids separated the thickened portal areas.

In the vicinity of a few lesions and occasionally elsewhere, liver lobules showed considerable reduction in size without other striking alteration (Fig. 2F). Central veins were approximately centrally located and collagen and reticulum stains showed no appreciable increase in connective tissue fibrils either within the lobules or about central veins or portal areas. There was, however, some disarrangement of the usual radial distribution of cell cords; only rarely was a necrotic liver cell seen in such areas.

In addition to the lesions just described, there were changes which were more widespread. The livers from guinea pigs killed

during the early part of the experiment showed fine droplet fat deposits in the parenchymal cells. In most, it was of moderate degree and distributed diffusely throughout the lobule. In a few, the fat was sharply limited to a zone around the portal areas. This was seen most often when the fat was present in only small amounts. Although fatty change was seen throughout the experimental period (35 weeks), it was occasionally absent and not infrequently was of slight degree, particularly in the last half of the experimental period.

Incomplete hyalinization of liver cell cytoplasm was often present but frank necrosis was limited to a few isolated cells or to very small groups of cells. In a few animals the small number of necrotic liver cells present bordered the portal area but in most livers the necrotic cells were irregularly distributed. In some livers, necrosis was not seen.

Portal cellular infiltrate, composed of lymphocytes and large mononuclear cells, was seen in most animals. When present it usually was of slight to moderate degree and in general was less frequent and less prominent in the latter part of the experimental period.

Proliferation of bile ducts or biliary epithelium was quite irregular, both as to occurrence and prominence. It was evident in less than one-half of the animals and usually was of slight to moderate degree. It was seen most frequently in the first part of the study.

The presence of increased amount of collagen in or about portal areas, although noted earlier in an occasional animal, was seen regularly after the sixteenth week. The collagen deposition was usually slight. In a number of livers after the sixteenth week, the portal areas were broader than normal and projected toward each other between the liver lobules. This tendency was in general progressive and in 4 animals thin trabeculae connected portal areas and surrounded liver lobules (Fig. 2E). Three of these occurred after the thirty-third week of the experiment. This trabeculation did not occur in all lobes and lobule involvement was variable in degree.

The portal changes described did not appear to bear any specific or consistent rela-

tionship to the lesions seen grossly. The portal involvement occurred in some livers showing no gross lesion and gross lesions were present in some lobes in which portal changes were absent or inconspicuous. Occasionally the portal alteration was somewhat more prominent in areas bordering the large lesions.

Discussion. The main purpose of this experiment, the production of clear-cut portal cirrhosis, was not achieved. In 4 of the guinea pigs, certain lobes or parts of lobes showed lobules surrounded by thin trabeculae formed partly of collagen. In a number of other livers, narrow cellular and fibrous portal extensions partly surrounded lobules in an irregular fashion. However, the process was not diffuse, and there was no striking disorganization of lobule architecture and arrangement. Since these latter features were missing, we do not feel justified in claiming the production of portal cirrhosis of the liver by the feeding of phosphorus. Many of these livers were thought of as showing precirrhotic changes. It is possible that frank cirrhosis might have resulted if the experiment had been continued for another six months.

This experiment throws little light on the working hypothesis that repeated zonal damage to the liver parenchyma will be followed by collagen deposition in the involved areas. This is true first because the necrosis was slight, inconstant in occurrence with only a tendency to be portal in location and second because intracellular accumulation of histologically demonstrable fat was often of slight degree and was periportal in location in only about one-half the livers studied.

This study cannot be compared to that of Mallory since in his experiment, in which cirrhosis was produced, rabbits were used almost exclusively. Our choice of the guinea pig as the experimental animal was made in order to avoid difficulties of interpretation which might arise in the rabbit due to the common presence of coccidial fibrosis.

The principal purpose of this paper is to report the occurrence of the focal lesions. We have not previously observed this type of injury following the chronic administration of a hepatotoxic agent.

The early pathogenesis of the focal lesions is not clearly evident. Localized acute necrosis of the parenchyma seems to us the most likely explanation. However, this was seen only in one liver. In evaluating this suggestion it should be borne in mind that cell debris resulting from acute necrosis is often removed from the site in 3 to 4 days. Also it would seem pertinent to recall here that the early necrosis phase of epidemic hepatitis was not observed in the cases reported by Lucké.³ An alternate explanation of the formation of these localized lesions would be the gradual loss of single or small groups of cells from an area. Against this theory is the fact that generally the entire lesion seemed to be of the same histologic age. Central fibrosis with the peripheral part of the lesion showing only collapsed sinusoids was not observed. It is true that the largest lesions occurred in the latter part of the study but these were considered as probably resulting from the development of adjacent rather than growth of individual lesions. It seems that the very gradual loss of cells from the lobule without replacement would lead simply to a reduction in size of the lobule, without sinusoidal collapse and condensation or proliferative fibrosis; this type of lesion was observed focally in a few livers.

Since histologically "early" lesions were seen throughout the experiment, it cannot be concluded that all lesions would progress to the end stage described above. The "early" lesions seen in the livers of animals killed in the latter part of the experimental period could be chronologically old lesions which had failed to undergo fibrosis. Against this alternative concept is the fact that in general the number of lesions per liver was greater in the second half of the experimental period and that lesions with extensive fibrosis were not seen before the twenty-third week.

A question raised by this experiment concerns the factor responsible for the localized nature of the lesions. It seems quite illogical to assume that the parenchymal cells in one part of the liver are inherently more suscepti-

ble to injury by phosphorus than another. It would appear that a more reasonable explanation could be based on some physiologic alteration of blood supply to the area. In an occasional superficial lesion sectioned vertically to the surface, the deep margin of the area of collapse was sharply limited by a fairly large hepatic artery (Fig. 2C). Primary vascular lesions were not observed.

Inasmuch as the hepatotoxic agent was administered by mouth, it is interesting that the gross lesions were neither limited to nor strikingly concentrated in the right or left half of the liver. From this observation, and reasoning from the theory that portal blood from different parts of the gastrointestinal tract reaches different lobes of the liver,⁴ it might be suggested that absorption of phosphorus in olive oil is not limited to one segment of the intestines. If the oil phosphorus mixture passed by the lacteals into the general circulation before reaching the liver, this suggestion would be invalid.

It is often difficult to determine whether fibrous tissue, which is seen in some areas where liver cells have disappeared and the sinusoids are collapsed, represents condensation fibrosis or newly proliferated collagen. In the present study we feel that some of the lesions showed amounts of fibrous tissue far beyond that which could be accounted for on a condensation basis. This belief is supported by the fact that the early lesions resembled the microscopic appearance of "acute yellow atrophy" with little or no increase in collagen, even though the sinusoids often were collapsed, whereas the few densely fibrotic lesions were seen only in the latter part of the study. In all likelihood the fibrosis occurred through both mechanisms.

Summary. In an attempt to produce portal cirrhosis, guinea pigs were fed white phosphorus in olive oil by mouth for periods up to 35 weeks. Microscopic examination of livers from animals killed after one week showed inconstant, slight to moderate cytoplasmic hyalinization and occasional to few necrotic liver cells. There was also moderate

³ Lucké, Balduin, and Mallory. *Tracy, Am. J. Pathol.*, 1946, **22**, 567.

⁴ Bartlett, F. K., Corper, H. J., and Long, E. R., *Am. J. Physiol.*, 1914, **35**, 36.

during the early part of the experiment showed fine droplet fat deposits in the parenchymal cells. In most, it was of moderate degree and distributed diffusely throughout the lobule. In a few, the fat was sharply limited to a zone around the portal areas. This was seen most often when the fat was present in only small amounts. Although fatty change was seen throughout the experimental period (35 weeks), it was occasionally absent and not infrequently was of slight degree, particularly in the last half of the experimental period.

Incomplete hyalinization of liver cell cytoplasm was often present but frank necrosis was limited to a few isolated cells or to very small groups of cells. In a few animals the small number of necrotic liver cells present bordered the portal area but in most livers the necrotic cells were irregularly distributed. In some livers, necrosis was not seen.

Portal cellular infiltrate, composed of lymphocytes and large mononuclear cells, was seen in most animals. When present it usually was of slight to moderate degree and in general was less frequent and less prominent in the latter part of the experimental period.

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Human red cells were agglutinated by *V. comma* (7 of 10), *S. pyogenes* (1 of 2), *Brucella bronchiseptica*, *Hemophilus pertussis*, and *Shigella alkalescens* (23).

Clumping of red cells by 7 of the strains of *V. comma*, one strain each of *H. pertussis*, and *S. pyogenes* was detectable only on microscopic examination. The suspension of *B. bronchiseptica* gave clumping clearly visible within 5 minutes by gross examination.

The addition of a saline suspension of *S. alkalescens* to human red cells caused clumping of the cells beginning in about 3 minutes. Microscopically, the cell aggregates resembled those produced by the action of isohemagglutinins. The speed of reaction and the degree of clumping of human cells by *S. alkalescens* was greater than that of other organisms studied. Among the bacteria studied, only *B. bronchiseptica* approached this intensity of agglutination. Further observations of the nature of hemagglutination by *S. alkalescens* are therefore presented.

Effect of Shigella alkalescens on red blood cells of various species. As shown in Table I saline suspensions of *S. alkalescens* N.I.H. No. 4 agglutinate the red cells of certain species of animals and do not agglutinate others.

Agglutination of human red cells by *S. alkalescens* No. 4 does not appear to be related to known factors in the red cells, such as blood agglutinogens A, B, O, M or N, and the Rh antigens, since differences would have been expected to occur among this sample of human cells.

Twenty-one strains* of *S. alkalescens* were

TABLE I.
Effect on Red Blood Cell of Several Species of Animals by the Addition of Saline Suspensions of *S. alkalescens* N.I.H. No. 4.*

Species	Type	No. of cell specimens	Result
Human	Group O Rh+	115	+
	" O Rh-	22	+
	" A Rh+	112	+
	" A Rh-	17	+
	" B Rh+	30	+
	" B Rh-	3	+
	" AB Rh+	3	+
	" AB Rh-	2	+
Monkey	(<i>M. rhesus</i>)	17	+
Hog		8	+
Horse		5	—
Dog		8	—
Cow		15	—
Sheep		2	—
Rabbit	New Zealand	37	—
Guinea pig		25	—
Mice	Swiss	5	—
Hamster		5	—
Rat	Wistar	5	—

* Mixtures of equal parts of 2% red cell suspensions and bacterial suspensions having turbidity equal to 500 p.p.m. silica standard, observed 1 hour at 35°C.

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tested against cells of man, horses, and hogs. All but one strain gave equally strong reactions with human cells; this weakly reacting strain gave definite microscopic agglutination. Against one specimen of hog cells one strain failed to cause clumping. This strain was a different one from that giving the weak reaction with human cells. All specimens gave negative results with a suspension of horse red blood cells.

Effect of various factors on the hemagglutinin of Shigella alkalescens. Broth cultures of *S. alkalescens* N.I.H. No. 4 were filtered through a Berkefeld filter, and the clear filtrate gave no reaction with human red cells. The possibility that the hemagglutinin was intimately associated with the bacterial cell was investigated.

A heavy suspension of *S. alkalescens* N.I.H. No. 4 in saline was exposed to sonic vibration at 8,000 oscillations per second for 20 min-

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cellular infiltration of portal areas and parenchymal fatty metamorphosis; the latter was sometimes periportal in location. These changes continued to be observed in most animals throughout the experimental period with increasing amounts of collagen in portal areas appearing as the experiment progressed. The increase in collagen was definite but never great. In 4 animals thin collagenous trabeculae connected portal areas and surrounded lobules. In a number of other animals fibrous extensions of portal areas were present but incomplete. There was neither prominent lobule distortion nor parenchymal hyperplasia. The failure to produce clear-cut portal cirrhosis is considered to be due to the incon-

stant and minimal degree of periportal necrosis.

After 9 weeks, the livers showed an increasing incidence of depressed gross lesions of irregular shape and size. The lesions in some livers were of such extent and severity as to produce marked deformity and shrinkage of various lobes. Microscopically, the lesions showed moderate to marked loss of parenchymal cells with collapse of sinusoids and reticulum stroma. In the latter part of the experiment a few such lesions showed marked fibrosis. The probable pathogenesis of these lesions is discussed and the incidence by lobes is given.

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Hemagglutination by Bacterial Suspensions with Special Reference to *Shigella alkalescens*.

JAMES J. GRIFFITHS. (Introduced by M. V. Veldee.)

From the Biologics Control Laboratory, National Institute of Health, Bethesda, Md.

Agglutination of red blood cells by various viruses has interested many workers, and has been found useful in immunological investigations of virus host relationships. Recently, Keogh, *et al.*¹ have reported agglutination of red blood cells by saline extracts of *Hemophilus pertussis*, *Hemophilus paraptussis*, and *Hemophilus bronchisepticus*. These authors suggest that this property of *Hemophilus pertussis* is related to the virulence of the organism and its ability to produce protective antibodies in mice.

In the course of an incomplete and unsuccessful survey of bacteria for Rh-like antigens using anti-Rh₀ blood typing serum, it was noted that saline suspensions of certain bacteria agglutinated human red cells in the absence of serum. It is the purpose of this report to describe some aspects of this phenomenon.

Experimental Observations. Hemagglutina-

tion by various bacterial species. Saline suspensions of bacteria grown for 24 hours on horsemeat infusion agar (and in a few instances on 5% rabbit blood agar) were prepared to have a density equal to 500 p.p.m. silica standard.² This type of suspension was used throughout these studies unless otherwise stated. Equal parts of these suspensions and 2% saline suspensions of once washed human Group O Rh-negative red cells were placed in small tubes. Agglutination was looked for after one hour's incubation at 35°C.

No agglutination of human red cells was observed using suspensions of the following bacteria (wherever more than one strain of organism was examined, the number of strains is given in parenthesis): *Aerobacter aerogenes*, *Alcaligenes fecalis*, *Bacillus anthracis*, *Brucella abortus*, *B. melitensis*, *Corynebacterium*

¹ Keogh, E. V., North, E. A., and Warburton, M. F., *Nature*, 1947, **160**, 63.

² Standard Methods for Examination of Water and Sewage, 6th Edition, American Public Health Association, New York, 1925, p. 4.

diphtheriae, *C. hektocnii*, *Diplococcus pneumoniae* (2), *Eberthella typhosa* (3), *Escherichia coli*, *Klebsiella pneumoniae* (2), *Lactobacillus acidophilus*, *L. bulgaricus*, *Pasteurella pestis*, *P. tularensis*, *Proteus vulgaris* (3), *P. morganii*, *Pseudomonas aeruginosa*, *Salmonella ballum*, *S. choleraesuis*, *S. enteritidis*, *S. paratyphi* (2), *S. schottmulleri*, *Serratia marcescens*, *Shigella ambigua*, *S. dispar*, *S. dysenteriae* (2), *S. flexneri* (14), *Staphylococcus aureus* (5), *Streptococcus fecalis*, *S. pyogenes* (1 of 2 strains), *S. salivarius*, and *Vibrio comma* (3 of 10).

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Monkey	(<i>M. rhesus</i>)	17	+
Hog		8	+
Horse		5	—
Dog		8	—
Cow		15	—
Sheep		2	—
Rabbit	New Zealand	37	—
Guinea pig		25	—
Mice	Swiss	5	—
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TABLE II.
Effect of Heat, Phenol, and Formalin on the Ability of *S. alkalescens* N.I.H. No. 4 to Agglutinate Human Red Cells.*

Treatment of suspension of <i>S. alkalescens</i> No. 4	Agglutination of human red cells Dilution of bacterial suspension						
	undil.	1:2	1:4	1:8	1:16	1:32	1:64
Fresh suspension	+++	++	++	+	+	±	—
37°C for 2 hr	+++	++	++	+	±	—	—
56 " " 5 min	+++	++	++	+	+	±	—
56 " " 15 min	+++	++	+	+	—	—	—
56 " " 30 "	++	+	—	—	—	—	—
93 " " 1 "	—	—	—	—	—	—	—
0.5% formalin 30 min	+++	++	++	+	±	—	—
0.5% phenol 30 min	+++	++	++	+	±	—	—

* Bacterial suspensions equal in turbidity to 500 p.p.m. silica standard. 2% saline suspensions of red cells.

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utes. Supernatant fluid from this suspension when undilute gave only microscopic reactions. Similar heavy suspensions of the organism were then rapidly frozen (in dry ice cellusolve mixture) and thawed 12 times, after which the material was placed in a Sharples centrifuge and spun at approximately 18,000 r.p.m. The supernatant resulting from this treatment gave reactions with human cells when diluted 256 times.

It was noted that human red cells could be washed 5 times with saline without affecting the degree of reaction with *S. alkalescens*. However, suspensions of *S. alkalescens* N.I.H. No. 4 washed with saline showed a progressive loss of hemagglutinin for human cells. Traces of hemagglutinin present after 3 washings disappeared after 6 washings.

These observations indicated that the hemagglutinin was separable from the bacterial cell, and therefore, the factor may have been adsorbed to the filter as previously used. Efforts to wash the material from Berkefeld filters were unsuccessful.

The effect of heat, phenol, and formalin on the agglutination factor of *S. alkalescens* is summarized in Table II.

Heat had an adverse effect on the hemagglutinin, causing slight loss of activity at 37°C for 2 hours, more noticeable effects at 56°C for 15 minutes, and destroying activity in one minute at 93°C. The addition of phenol and formalin to bacterial suspensions appeared

to lessen the activity of the hemagglutinin after 30 minutes contact.

Agglutination of human red cells by *S. alkalescens* took place at 5°C and at 56°C as readily as at room temperature.

The production of hemagglutinin by cultures of *S. alkalescens* at 35°C is shown in Table III. Bacterial suspensions of comparable turbidity prepared on various days of growth on agar medium showed that hemagglutinin increased from its level at 2 days to maximum proportions in 5 to 7 days, and thereafter fell in titer.

The possibility that agglutinins for human cells present in various animal serums and tissue juices may have influenced the reactions observed, appeared less probable when *S. alkalescens* grown on a medium containing a pancreatic digest of casein gave equally good reactions.

Antiserums, prepared by injecting formalized suspensions of *S. alkalescens* into rabbits, agglutinated the organisms. The agglutinated organisms washed free of serum failed to agglutinate human cells.

A phenomenon not well explained was observed in the course of this study. Twenty-three strains of *S. alkalescens* on horsemeat infusion agar were placed in a cold room (5°C) for approximately 6 weeks. At this time, transplants were prepared and from these actively growing organisms suspensions were made, none of which agglutinated human

TABLE III.

Titration of Hemagglutinin in Suspensions (Turbidity = 500 p.p.m.) of *S. alkalescens* on Different Days of Growth*

Day of growth	Agglutination of human red cells Dilution of bacterial suspension							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
2nd	++	++	+	—	—	—	—	—
3	+++	+++	++	+	—	—	—	—
5	+++	+++	++	++	++	+	—	—
6	+++	+++	++	++	++	+	±	—
7	+++	+++	++	+	+	+	+	—
8	+++	++	+	+	+	±	—	—
9	+++	++	+	±	—	—	—	—

* Bacterial suspension prepared each day from growth of *S. alkalescens*. Turbidity = 500 p.p.m. silica standard. 2% saline suspensions of red cells.

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red cells. Additional dried cultures (such as were used originally in these studies) were obtained and fresh transplants from the dried cultures showed agglutinating activity as noted before. Thus it would appear that the hemagglutination factor is labile and under certain conditions may disappear from strains known to have possessed this factor.

Discussion. The phenomenon of hemagglutination by bacteria as reported here appears to be different from reactions reported by Huebner, Thomsen, Friedenreich,³ and Terada.⁴ These authors observed that red cell suspensions in which certain strains of bacteria (*Corynebacterium* and gram-positive cocci) are allowed to grow become agglutinable with all human serums used to test the red cells. Nor does the phenomenon reported here appear to be related to the action of *Corynebacterium hektocnii*,⁵ or a mustard bacillus,⁶ which impart to certain serums in which they are grown the ability to agglutinate all human and some animal red cells.

The ability of bacterial suspensions to agglutinate red blood cells reported by Kraus and Ludwig⁷ in 1902 and later elaborated by

Guyot⁸ and Fukuhara⁹ appears to have received little attention.¹⁰ Kraus and Ludwig reported that certain strains of staphylococci and vibrio agglutinated rabbit cells. They reported that hemagglutination by organisms could be interfered with by the action of specific anti-serum. Guyot reported that 12 of 18 strains of organisms designated *B. coli* agglutinated the cells of various animals. Eight of these strains showed considerable difference in the species of animals with which they reacted. He was unable to separate hemagglutinin from the bacterial cell. Fukuhara found that diphtheria, mouse typhoid, paratyphoid, and Friedlander's bacilli and *Sarcina* contained hemagglutinins for cells of some species of animals. He extracted the hemagglutinin with alcohol from bacterial suspensions treated with 0.1N NaOH. The possibility that such reactions might be used in immunological studies of bacteria, as suggested by Keogh *et al.*, gives bacterial hemagglutinin added significance.

The finding of an active hemagglutinin in cultures of *S. alkalescens* provides a readily grown source of hemagglutinin for the study of the phenomenon of agglutination. In ad-

³ Friedenreich, V., *The Thomsen Hemagglutination Phenomenon*, Copenhagen, 1930.

⁴ Terada, K., *Tairan Iyakkai Zasshi*, 1936, **35**, 1267.

⁵ Davidsohn, L., and Tobarsky, B., *J. Inf. Dis.*, 1940, **67**, 25.

⁶ Grove, E. F., and Crum, M. J., *J. Lab. and Clin. Med.*, 1930, **16**, 259.

⁷ Kraus, R., and Ludwig, S., *Wien. Klin. Wchnschr.*, 1902, **15**, 120.

⁸ Guyot, G., *Chl. f. Bakteriologie*, 1908, **47**, 640.

⁹ Fukuhara, Y., *Z. f. Immunitätsforsch.*, 1909, **2**, 313.

¹⁰ Pearce, R. M., and Winne, C. K., *Am. J. Med. Sci.*, 1904, **128**, 669.

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Agglutination of human red cells by *S. alkalescens* took place at 5°C and at 56°C as readily as at room temperature.

The production of hemagglutinin by cultures of *S. alkalescens* at 35°C is shown in Table III. Bacterial suspensions of comparable turbidity prepared on various days of growth on agar medium showed that hemagglutinin increased from its level at 2 days to maximum proportions in 5 to 7 days, and thereafter fell in titer.

The possibility that agglutinins for human cells present in various animal serums and tissue juices may have influenced the reactions observed, appeared less probable when *S. alkalescens* grown on a medium containing a pancreatic digest of casein gave equally good reactions.

Antiserums, prepared by injecting formalized suspensions of *S. alkalescens* into rabbits, agglutinated the organisms. The agglutinated organisms washed free of serum failed to agglutinate human cells.

A phenomenon not well explained was observed in the course of this study. Twenty-three strains of *S. alkalescens* on horsemeat infusion agar were placed in a cold room (5°C) for approximately 6 weeks. At this time, transplants were prepared and from these actively growing organisms suspensions were made, none of which agglutinated human

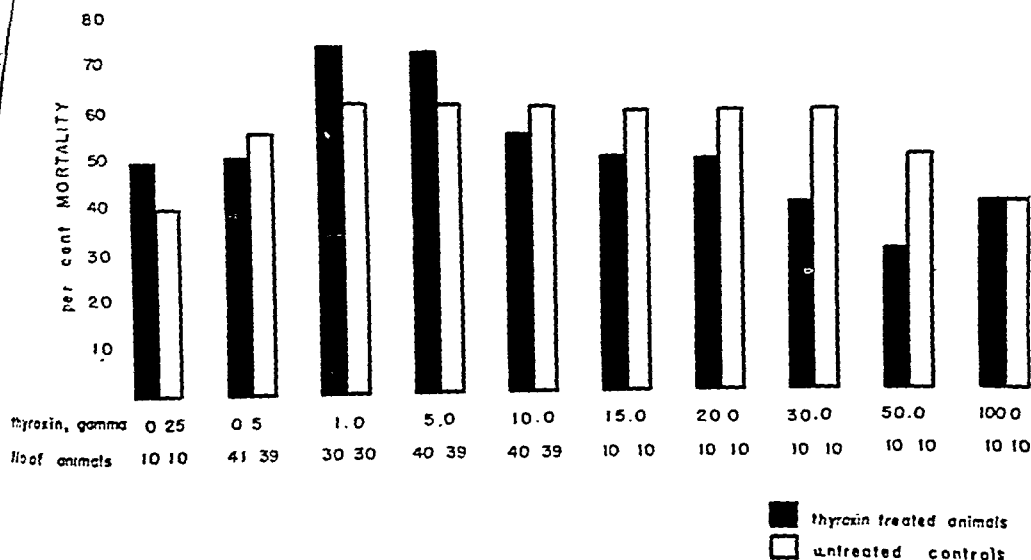


FIG. 1.
Effect of Various Doses of Thyroxin on Mouse Poliomyelitis.

experiments were of the AB strain and came from the colony of Dr. John Bittner, University of Minnesota. Their food consisted of Purina fox chow. The experiments were carried out during the months of March, June, and July.

Fifty mg of thyroxin crystals containing not less than 64% iodine were dissolved in 5 drops of N sodium hydroxide and diluted with distilled water to 50 cc. Injections of thyroxin were made subcutaneously four days before the inoculation with the virus so that the height of the thyroxin effect would coincide with the end of the incubation period.

The MM mouse virus was obtained from Dr. Raymond Bieter, University of Minnesota. 0.1 cc of a 10^{-6} dilution of the virus preparation was injected intraperitoneally without anesthesia. The same dilution was used throughout the experiments.

All animals which showed signs of paralysis and encephalitis or were found dead within the period of 24 hours to 2 weeks after the injection of the virus were considered to have succumbed to the disease.

The results in Fig. 1 show that no dose of thyroxin has been found which could protect

TABLE I.
Mortality of Thyroxin-treated Animals and Untreated Controls.

	Thyroxin-treated animals	Non-treated controls
% mortality after one week	35.0	39.7
% mortality after 2 weeks	51.2	55.6
No. of animals	211	207

the animals to a significant degree. Whenever differences in the susceptibility to the disease between the thyroxin-treated and untreated control animals seemed to occur, these differences were found insignificant when a larger number of animals were used.

Table I summarizes the result of all experiments on thyroxin-treated and untreated control animals. No change in the length of the incubation period and no significant difference in the mortality between thyroxin-treated and untreated animals could be observed.

Summary. The susceptibility of young mice of the AB strain to poliomyelitis due to the MM virus could not be affected significantly by treatment with various doses of crystalline thyroxin.

dition, the presence of hemagglutinin may be useful in distinguishing this bacterial species from others of the genus *Shigella*.

The specificity of the action of *S. alkalescens* on human, monkey, and hog red blood cells appears to indicate that some substances common to these three species are present in their red cells, while absent in cells of other animal species. This specificity is in rather marked contrast to the findings of Guyot, and also to the recent report of Keogh.

Summary. 1. Saline suspensions of *S. alkalescens*, *B. bronchiseptica*, *H. pertussis*, *V. comma*, *S. pyogenes* are capable of clumping

human red blood cells.

2. Suspensions of *S. alkalescens* agglutinate human, monkey, and hog red blood cells, and do not agglutinate cells of certain other animal species.

3. The hemagglutinin of *S. alkalescens* can be separated from the bacterial cell by high speed centrifugation after successive freezing and thawing of bacterial suspensions.

4. The hemagglutinin of *S. alkalescens* is labile, being destroyed by heat, and spontaneously disappears from cultures on standing at 5°C for 6 weeks.

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Effect of Thyroxin on Mouse Susceptibility to Polioencephalitis.*

FRANK GOLLAN. (Introduced by M. B. Visscher.)

From the Department of Physiology, University of Minnesota, Minneapolis.

The fact that a small fraction of persons in a population group display recognizable symptoms of poliomyelitis even in epidemic situations has led to the suggestion that there may be physiological factors involved in determining the susceptibility to the disease.

The higher morbidity rate among children and pregnant women and the possibility of predisposing factors such as physical exertion and preceding infections suggest that this hypothetical physiological factor may be of an unspecific character. An increased metabolic rate is common to all these conditions mentioned. However, it may be noted that the observation of general decreases in BMR during the warmer season of the year when most poliomyelitis epidemics occur is not in agreement with the assumption of an increased metabolic rate as a factor increasing the susceptibility to poliomyelitis.

Recent experimental studies¹ on the influence of environmental temperature on the resistance of Swiss white mice to the polio-

myelitis virus showed a marked tolerance of these animals to the infection when acclimated to low temperatures. Since thyroxin secretion is increased upon exposure to cold the influence of thyroid treatment on the susceptibility to poliomyelitis was investigated. On a small number of Swiss white mice it could be shown² that thyroactive substances were able to prolong the incubation period of poliomyelitis up to 100% whereas thiouracil had the opposite effect.

The effect of the thyroid hormone on the resistance to neurotropic virus disease is of great theoretical and practical importance and therefore further studies on this subject seemed indicated. Our investigations were concerned with the effect of crystalline thyroxin on the susceptibility of mice to the MM strain of mouse polioencephalitis. Thyroxin was chosen instead of other thyroactive extracts or substances because the effect of crystalline thyroxin on the metabolism lends itself in a more accurate way to a standardization of experimental conditions.

The 3-5-weeks-old mice used for these

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¹ Holtman, D. Frank, *Science*, 1946, 103, 137.

² Holtman, D. Frank, *Science*, 1946, 104, 50.

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16 Cox, H. R., van der Scheer, J., Aston, St., and Bohnel, E., *J. Immun.*, 1947, **50**, 149.

17 Jungeblut, C. W., and Dalldorf, G., *Am. J. Publ. Health*, 1943, **33**, 169.

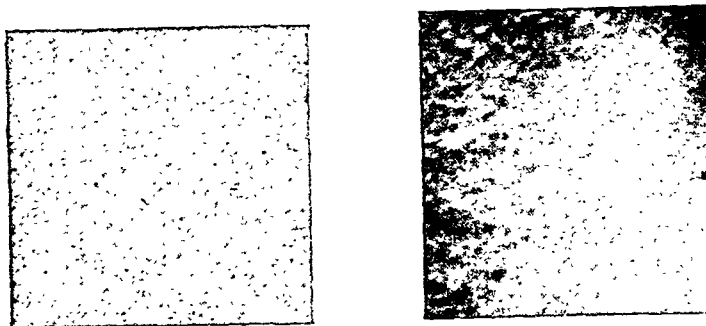


FIG. 1.

Electron micrographs and shadowgraphs of an infected mousebrain suspension containing 3.9×10^{-14} g nitrogen per LD₅₀. Magnification 30,000.

ticle dimensions could be made by measuring the shadow width and shadow length. The shadow length should be 5 times the diameter.

In both micrographs there is an indication for a slight asymmetry of the particles. In measuring the diameter of the particles a minimum dimension of 10 mμ and a maximum of 20 mμ with an average of about 12 mμ was found.[†]

These micrographs confirm the findings of

[†] These measurements would indicate a molecular weight of the order of 500,000 to 1,000,000.

Loring, Marton and Schwerdt² of the absence of thread-like particles³ and show also essentially the same shape and size of particles as described by these authors.

Summary. Electronmicroscopic studies of a preparation of purified MM Poliomyelitis virus show an uniformity of particle size in the range from 10 to 20 mμ with an average diameter of about 12 mμ.

² Loring, H. S., Marton, L., and Schwerdt, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 291.

³ Gard, S., *Acta Med. Scand.*, Suppl., 1943, **143**, 173.

16308

Serum and Plasma Antithrombin.*

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For years it has been known that blood has the ability to inactivate added, as well as spontaneously formed, thrombin. Rettger¹ attributed this to the plasma proteins as a specific function; Lenggenhager² related the activity to the albumin fraction. Smith's³ group has suggested that the *rate* of thrombin

destruction varies with the heparin concentration and that the absolute *amount* is limited by the concentration of heparin "cofactor." Wilson⁴ developed a method for estimating antithrombin by so diluting plasma

¹ Rettger, L. J., *Am. J. Physiol.*, 1909, **24**, 496.

² Lenggenhager, Karl, *Helv. med. Acta*, 1935, **1**, 527.

³ Seegers, W. H., Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Science*, 1942, **96**, 300.

⁴ Wilson, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 676.

* Abridgment of a portion of thesis, submitted by Dr. Owen to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D. in Medicine.

Table I. In this experiment a 43-fold purification was achieved and after the deduction of the 35% loss of virus activity a purification factor of about 28 resulted.

Conclusion. A simple method requiring

standard laboratory equipment only is described which by means of freezing and thawing, alcohol precipitation and dialysis accomplishes a marked purification of the MM poliomyelitis virus.

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Electronmicroscopy of the Purified MM Poliomyelitis Virus.*

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With the technical assistance of Miss Virginia Mary Kletzin.

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The purified virus used for electronmicroscopic studies was prepared in essentially the same way as described in detail in the preceding paper. During the preliminary studies the observation was made that highly concentrated brain suspensions in phosphate buffer yielded heavier precipitates after freezing and that a higher degree of purification could be achieved by keeping the protein concentration as high as possible.

A 25% mixture of infected mouse brain and cord in 0.1 M phosphate buffer pH 7.0 was prepared by homogenizing the previously frozen tissue in a Potter-Elvehjem glass homogenizer. The mixture was then frozen for 24 hours. After thawing the tissue particles were centrifuged off, the clear supernate was extracted with two-thirds its volume of ether, centrifuged and the gelatinous layer at the surface was discarded. The remaining ether was removed in a desiccator and the solution was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The temperature was then lowered to -1°C and enough chilled methyl alcohol was added to a final concentration of 30% methanol. After 6 hours the precipitate was centrifuged off, washed once with 30% methyl alcohol in buffer, suspended in one-tenth of the original volume, the insoluble particles were centrifuged off and the supernate was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The super-

natant fluid was dialysed against distilled water for 24 hours, the water insoluble proteins were centrifuged off and discarded. The supernatant fluid was frozen for 2 weeks, thawed and a final precipitate was centrifuged off and discarded.

By this procedure the nitrogen content was reduced from 2.72 mg per ml in the original clear solution to 0.0119 mg per ml in the final fraction. Thus, a 228 fold purification was achieved or 99.5% of the total nitrogen of the original solution was removed. The final yield amounted to about 10% of the total virus activity of the original solution and therefore a purification factor of about 22.8 resulted. The final fraction contained 1.19×10^{-12} g of "virus" nitrogen per ml and one inoculum contained 3.9×10^{-14} g of "virus" nitrogen.

The mounts for the electron micrographs were prepared by the usual technique of applying a small drop of the 10 times diluted final fraction (1.19×10^{-13} g nitrogen per ml) to a thin formvar membrane supported by a $\frac{1}{8}$ inch diameter disc of 200 mesh copper screen and allowing it to dry. The gold-shadowed specimen was prepared by applying a small drop of the solution to a microscope slide. This was treated by the gold shadowing technique of Williams and Wyckoff¹ (8 AU film of gold at a shadow angle of 5 to 1) stripped with collodion and mounted on the copper screen. Measurement of the par-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Williams, R. C., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 265.

TABLE I.
Antithrombic Activity of Plasmas and Serums
from Two Dicumarol-treated Dogs (A and B).

% prothrombin in plasma*		% thrombin deterioration in 60 seconds	
A	B	Plasma	Serum
100 (320 units per ml)		59	32
	100	61	36
	77	64	43
71		60	35
	66	67	38
52		68	45
	45	60	54
43		63	53
	33	66	58
31		68	56
	24	62	58

* Two-stage titration.⁷

TABLE II.
Antithrombic Activity of Plasma at Various
Stages During Spontaneous Clotting.

Time after collection of blood, min	% prothrombin*	% thrombin deterioration in 60 sec.
Immediately	100 (305 units per ml)	61
3	68	53
5	52	48
7†	36	44
9†	21	40
11†	3	33
15†	1	33

* Two-stage titration.⁷

† Fibrinogen-free.

the high prothrombin samples. From study of the oxalated plasmas alone, no difference was seen.

Somewhat similar results are noted in the following experiment (Table II). Fifty milliliters of normal dog blood were collected; at intervals 4.5 ml were added to 0.5 ml of 1.85% potassium oxalate. Clotting began at about the sixth minute; thus the last 4 specimens were fibrinogen-free. It would seem that serum has an antithrombic activity varying inversely with the amount of thrombin to which it has been exposed during clotting.

If this is correct, addition of sufficient thrombin to plasma should simulate the effect of spontaneous clotting. One milliliter of oxalated plasma was mixed with 1.0 ml of 500 units per milliliter thrombin. In 2 hours no thrombin was demonstrable. After appropriate dilution the antithrombic activity of this solution was less than that of control serum: thrombin-treated plasma, 14% inactivation in 60 seconds; serum, 30% (Fig. 1).

Summary. A method of estimating the rate of natural antithrombic activity is suggested. When this method was used, dog plasma, regardless of the prothrombin level after dicumarolization, seemed to exert about the same antithrombic activity. On the other hand, dog serum antithrombin tended to vary inversely with the prothrombin content of its original plasma, and serum obtained from blood containing little prothrombin was antithrombic to about the same extent as plasma. These observations suggest that antithrombic activity be determined on plasma unless due consideration is given to the antithrombic activity already performed by serum.

set aside for development of serum. Table I shows the antithrombic activity of comparable plasmas and serums from 2 dogs. If antithrombic determinations had been made on serums alone, the evidence would suggest more antithrombin in serums from the low prothrombin samples than in serums from

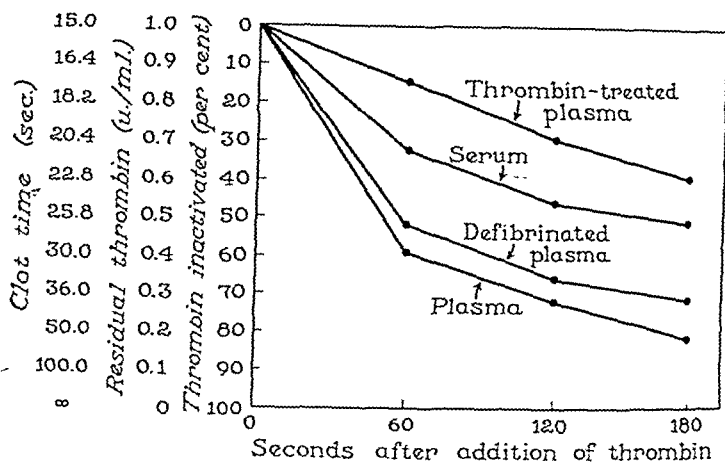


Fig. 1.
Deterioration of thrombin added to diluted serum or plasma.

that it inactivates 1 unit of thrombin in 4 minutes. Astrup and Darling,⁵ adding an excess of thrombin to a small amount of plasma, await maximal inactivation before measuring the residue. By both methods no difference in the antithrombic activity of serum and plasma was detected, in spite of the fact that serum inactivates about 300 thrombin units per milliliter in its development.

In an attempt to increase the sensitivity of measurement of thrombin inactivation, the following method was devised. Serum or oxalated plasma is diluted tenfold with buffered saline solution (1 part isosmotic imidazole buffer,⁶ 9 parts 0.9% sodium chloride). To 1.0 ml of this solution is added 0.1 ml of thrombin (11 units per milliliter), to make a final thrombin concentration of 1 unit per milliliter. In the case of plasma, fibrin begins to form in 20 to 30 seconds; sharp agitation of the tube clumps the fibrin and prevents its obscuring the final step. Exactly 60 seconds after the addition of thrombin, and in some cases at intervals thereafter, 0.4 ml of the solution is added to 0.1 ml of 0.5% buffered fibrinogen solution, and the coagulation time is determined. The

amount of residual thrombin is calculated by comparison of the clot times with the times found for dilutions of thrombin with buffered fibrinogen solutions (Fig. 1).

Tests were performed at 24° to 26°C. Parke-Davis beef thrombin and Armour's Fraction I (fibrinogen) were routinely employed, but were checked against homologous preparations, with negligible variation in the results.

By the use of this method it was found that oxalated plasma (diluted 1:10) from 12 normal dogs inactivated about 60% (40 to 82) of the added thrombin in 60 seconds (Fig. 1). Serum (diluted 1:10) from the same blood samples inactivated about 25% (14 to 38) in 60 seconds. Oxalation of the serum, or preliminary defibrination of the plasma by the addition of only a fraction of a unit of thrombin, altered the results only slightly. The significance of the amount of thrombin deterioration may be estimated from this experiment: a 1:2 dilution of pooled normal dog serum inactivated 81% of added thrombin in 1 minute; 1:3 serum, 67%; 1:4 serum, 50%; 1:5 serum, 39%; 1:6.7 serum, 28%; 1:10 serum, 23%; 1:20 serum, 10%; 1:40 serum, 7%; and 1:100 serum, trace.

To examine the serum-plasma difference further, dicumarol was administered intravenously to dogs; blood samples were collected periodically. Along with each sample preserved by oxalate, another specimen was

⁵ Astrup, Tage, and Darling, Sven, *Acta physiol. Scandinav.*, 1942, 4, 293.

⁶ Mertz, E. T., and Owen, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, 43, 204.

⁷ Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, 114, 667.

TABLE I.

Kidney Alkaline Phosphatase Determinations in Normal Rats and in Alloxan Diabetes.
Results Expressed as mg Phosphorus Released per Gram Tissue Solids per Hour.

Normals		Alloxan diabetics			
Rat No.*	mg P†	Rat No.*	Time after alloxan, days	Blood sugar, mg %	mg P†
1a	26	8a	7	—	2.0
2	25	8b	7	—	1.7
3	17	9a	11	—	5.1
4a	62	10	12	500	1.9
4b	66	11†	21	—	27.48
5a	33-45	12†	21	602	10.14
5b	47-66	13†	21	612	12.21
6	22-34	14a	42	764	12.17
7	27-45	14b	42	764	13.17

* Nos. with *a* or *b* indicate assays of individual kidneys. Other numbers refer to pooled kidney analyses.

† Where 2 figures are given, the first is the result obtained without added Mg; the second with added Mg. Where only one figure is given, no Mg was added.

‡ Animals 11, 12, and 13 received no insulin. Others of this alloxan-treated group received 2-3 units per day until 3-4 days before animals were sacrificed for analysis.

TABLE II.

Rat Kidney Alkaline Phosphatase Determinations in Experimental Hypo- and Hyperglycemia
Expressed as mg P Released per g Tissue Solids per Hour.

Hypoglycemics			Hyperglycemics		
Rat No.*	Blood sugar, mg %	mg P†	Rat No.*	Blood sugar, mg %	mg P†
15a	33	26.50	17a	800	25.45
15b	33	32.49	17b	800	23.38
16	40	26.41	18	1024	23.37
			19	888	23
			20a	812	27.40
			20b	812	27

* and † have the same meaning indicated in Table I.

to provide data for calculating the dry weight of the portion extracted. Aliquots weighing 30 to 80 mg were extracted for 24 hours at 4°C in 5 ml distilled water. The extract used for phosphatase assay was freed of the residue by centrifugation.

Incubation of enzyme with substrate. The substrate consisted of sodium β -glycero-phosphate (concentration 0.5%)⁴ at a pH of 8.6 with and without the addition of 0.009 M MgCl_2 . The incubation of the filtrate with the substrate and the determination of the inorganic phosphorus liberated were carried out according to the procedure described by Shwachman.⁴ In the present study, the amount of phosphorus hydrolyzed was related to the dry weight of the tissue.

The average variation in phosphorus liberated by different aliquots of the same enzyme sample for 13 consecutive pairs of samples was 4%; the range for all but one was 0-6%, the exception being 14%. The average variation between the extracts of the 2 kidneys when determined separately was 11.9% for 7 pairs, with a range of 0.8 to 25%. The values of phosphatase activity obtained with added magnesium plotted against the values without magnesium showed a linear relationship, corresponding to the

$$\text{ratio} \frac{\text{activity with Mg}}{\text{activity without Mg}} = 1.7.$$

Histochemical methods employed. After fixation of the tissues in cold 80% alcohol and subsequent imbedding in paraffin, the

⁴ Shwachman, H., *J. Ped.*, 1941, 19, 38.

sections were incubated at 37°C in glycerophosphate at pH 9.4 for 3 and 6 hours according to Dempsey and Deane's modification of Gomori's method.⁵ Hematoxylin eosin preparations were also made from the same blocks.

Results. The results on the normal and alloxan diabetic rats are presented in Table I, and those on the hypo- and hyperglycemic rats in Table II. The normal rat kidneys released an average of 36 mg P per g of tissue per hour. When the abnormally high results on the kidneys of rat No. 4 were excluded, the average was 28 without Mg, and 47 with Mg. All but one of the alloxan diabetic rats had abnormally low phosphatase activity. Those sacrificed 7 to 12 days after alloxan administration had activities of 5 or less; 3 of the 4 rats allowed recovery periods of 21 to 42 days had activities of 10-13 without Mg, and 14-21 with Mg. Rat No. 11 had a normal phosphatase activity 21 days after the alloxan administration. All of the rats whose blood was analyzed showed marked hyperglycemia.

Neither hypoglycemia nor hyperglycemia, maintained for the relatively short periods of our experiments, had any demonstrable effect on the phosphatase activity of the kidneys (Table II).

There were no qualitative histochemical differences in alkaline glycerophosphatase between the kidneys of normal and of acutely hyperglycemic and hypoglycemic animals. However, in the rats given alloxan 1 to 6 weeks before autopsy, there was a greatly reduced amount of phosphatase in the epithelium of groups of proximal convoluted tubules in which the cells were flatter and less eosinophilic than average. These tubules were particularly numerous in the subcapsular region and unquestionably represented regenerated tubules that had replaced those damaged by alloxan. In addition, small formless intratubular masses were present in the medulla. These masses were stained by hematoxylin and were frequently covered by epithelium. They appeared to consist of ne-

crotic and calcified clumps of epithelium and were more common in the kidneys of rats killed soon after receiving the dose of alloxan than in rats killed later.

A recent paper⁶ reports a drop in the phosphatase content of the kidney in 6 to 72 hours after administration of alloxan, which it ascribes to renal damage. Bennett and Behrens⁷ have shown that the elevation in non-protein nitrogen following alloxan is roughly proportional to the dose, is independent of hyperglycemia, is not altered by insulin, and may persist for as long as 31 to 34 days after administration of the drug; and further, that it is correlated with the "histopathological" changes in the kidneys. Breedis, Florey and Furth⁸ have reported that, following administration of a nephrotoxic agent, such as uranium nitrate, the reappearance of phosphatase in the regenerating tubules is slow, not attaining a normal value in 25 days. In the present study, 1 of 3 rats exhibited a normal amount of phosphatase after 21 days, a fourth examined after 42 days still showed a low phosphatase activity.

Summary. 1. The alkaline phosphatase activity of the rat kidney, which reflects principally the enzyme content of the proximal convoluted tubules, could not be changed from the normal by varying the amount of glucose for reabsorption over periods of 6 to 12 hours.

2. The kidneys of rats suffering from chronic alloxan diabetes as a rule contained less than normal amounts of alkaline phosphatase. The lowest values were obtained when first examined one week after the dose of alloxan, but recovery of a normal content was not complete when the last examination was performed after 42 days. The proximal convoluted tubule epithelium that had regenerated after the alloxan injury was thus observed to be slow in acquiring its normal enzyme content.

⁶ Menten, M. L., Janouch, M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 33.

⁷ Bennett, L. L., and Behrens, T., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 5.

⁸ Breedis, C., Florey, C. M., and Furth, J., *Arch. Path.*, 1943, **36**, 402.

⁵ Dempsey, E. W., and Deane, H. W., *J. Cell. Comp. Physiol.*, 1946, **27**, 159.

Side Reactions to Pyribenzamine Medication.

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A number of recent publications have dealt with the use of the new anti-histaminic drugs, and more are appearing every month. Most of these articles mention various side effects encountered, but to date the author has seen no reports of controlled observations of such side effects. The following data regarding pyribenzamine may therefore be of interest.

All the patients who were studied were receiving injections of typhoid vaccine, and the effect of pyribenzamine upon their reactions to the vaccine will be reported elsewhere. At the time of administration of the vaccine, each patient was given either pyribenzamine (PBZ) tablets or indistinguishable placebos,* with instructions as to dosage. Forty-eight adults were on 100 occasions given five 50-mg PBZ tablets with instructions to take one tablet at once, one every 4 hours throughout the day, and one tablet the following morning. Forty-nine subjects were on 102 occasions given placebos with the same instructions. Thirty-seven subjects on 56 occasions were given ten 50-mg tablets of PBZ with instructions to take 2 tablets at a time instead of one. Each subject had a report sheet on which he was asked to indicate whether or not he had

experienced any of the symptoms listed on the sheet. The accompanying table shows the percentage occurrence of the symptoms in the 3 groups of subjects.

These data indicate that the smaller dose of PBZ used here, which is the dose most frequently used in treating allergic conditions, has a negligible effect in producing the stated symptoms, all of which have been ascribed to PBZ medication. For example, drowsiness is the most commonly reported side reaction to PBZ, but the administration of 250 mg in 24 hours gave an incidence of drowsiness of only 37% as compared with 30% for placebo medication. The 2 patients who were bitter in their complaints of drowsiness both received placebos. Nausea, dizziness, and insomnia were somewhat more frequent in the 250 mg group as compared with the controls. Headache was less frequent, and nervousness and dryness of the mouth were apparently equally frequent in both groups. Doubling the dose of PBZ was accompanied by a definite increase in the incidence of symptoms, but headache was still less frequent than among the controls.

These data indicate the importance of using controlled studies in evaluating the side reactions, as well as the therapeutic effects, of a new drug.

Summary. A controlled study was made of side reactions to pyribenzamine. Five 50-mg tablets were given over a 24-hour period to 48 subjects; some subjects were used 2 or 3 times, and the total number of times the drug was given was 100. Placebos were given to 49 subjects a total of 102 times. The dosage of pyribenzamine was doubled in 37 other subjects on 56 occasions. Nervousness, dryness of the mouth, and headache were more frequent in the control group as compared with the smaller dose group of pyribenzamine. Drowsiness, nausea, dizzi-

TABLE I.
Percentage Occurrence of Side Effects.

Symptom	Five 50-mg PBZ tablets in 24 hr (N = 100)	Ten 50-mg PBZ tablets in 24 hr (N = 56)	Placebos, 5 in 24 hr (N = 102)
Drowsiness	37	48	30
Headache	26	36	42
Nausea	17	23	8
Dizziness	24	41	15
Nervousness	13	21	15
Dryness of mouth	29	45	30
Insomnia	12	23	6

* The pyribenzamine and placebos used were supplied by the Ciba Pharmaceutical Company, Summit, N.J.

ness and insomnia were less frequent in the control group. All side reactions mentioned occurred more frequently when the dose of pyribenzamine was doubled.

This study was prepared at Walter Reed General Hospital while the author was in the Army Medical Corps.

16311

Protective Action of Vitamins C and P Against Dichlorophenarsine Hydrochloride (Clorarsen).

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This study was undertaken specifically to ascertain whether the use of vitamins C and P in combination would protect against the acutely toxic effect of intravenously injected dichlorophenarsine hydrochloride in mice. It was thought that if these vitamins exerted a favorable effect on capillary fragility it might be demonstrated by the manifestation of a decrease in the acute toxicity of arsenic, since arsenic is a potent capillary poison.

The literature dealing with decreased capillary resistance as one of the toxic manifestations of antisyphilitic therapy has been reviewed by Horne and Scarborough.¹ The apparently protective action of vitamin C against the toxicity of arsenicals has been commented on by several groups of authors.^{2,3,4} Scarborough and Stewart have reported that vitamin P (hesperidin) treatment increased capillary resistance in erythema and dermatitis due to arsenic and bismuth.⁵

Goldforb⁶ suggested that the vitamin in

the form of aqueous extracts of whole lemon might prevent arsenical encephalopathy in patients receiving intensive arsenical therapy. And, Goldstein, Stalman and Goldforb⁷ found that treatment with the methyl chalcone of hesperidin decreased the mortality of a standard dose of mepharsen in rabbits from 90 to 57%, a difference that was of questionable significance ($X = 2.9$).

In a preliminary study on groups of control and treated mice (Table I), it was found that vitamin C alone (2.2 millimoles per millimole of dichlorophenophenarsine hydrochloride) had a favorable, but not statistically significant effect. The same was true when similar groups of mice were treated with hesperidin methyl chalcone alone (1 mg each daily for 9 days previous and 3 days following the injection of the arsenical).

For these reasons it was decided to investigate the protective effect of a combination of these two substances on the acute toxicity of a standard dose of intravenously administered dichlorophenarsine hydrochloride.

Method. Adult female white mice, weighing from 17 to 24 g (average weight, 21 g apiece in each group) maintained on a diet of Purina Checkers plus lettuce were used. Injections of dichlorophenarsine hydrochloride (clorarsen, Squibb) dissolved in saline were made into the tail vein, and all animals were observed for 3 days after injection, since those not

¹ Horne, G., and Scarborough, H., *Lancet*, 1940, 2, 66.

² Sulzberger, M. B., and Oser, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1935, 32, 716.

³ Bundesen, H. N., Aron, H. C. S., Greenbaum, R. S., Farmer, C. J., and Abt, A. F., *J. Am. Med. Assn.*, 1941, 117, 1692.

⁴ McChesney, E. W., Barlow, O. W., and Klinek, G. H., *J. Pharm. and Exp. Therap.*, 1944, 80, 81.

⁵ Scarborough, H., and Stewart, C. P., *Lancet*, 1938, 2, 610.

⁶ Goldforb, A. E., *Arch. Dermat. and Syph.*, 1941, 43, 536.

⁷ Goldstein, D. H., Stalman, A., and Goldforb, A. E., *Science*, 1943, 98, 245.

TABLE I.
Results Showing the Protective Action of Vitamins P and C Given in Combination Against the Toxicity of Dichlorophenarsine Hydrochloride.
(Group treated with a combination of Vitamins C and P.)

Group No.	Control groups				Lot of hesperidino methyl chalcone	Vitamin C alone			Statistical significance
	No. of mice	No. dead	No. survived	% survival		No. of mice	No. dead	% survival	
I	40	36	4	10	No. 1369	7	33	82.5	$\chi^2 = 39.1$
II	20	17	3	15	" 1369	3	12	80.0	$\chi^2 = 14.7$
Subtotal Lot No. 1369	60	53	7	11.6		10	45	81.8	$\chi^2 = 57.0$
III	16	9	7	44	" 1653	0	17	100	$\chi^2 = 11.9$
IV	20	11	9	45	" 1653	8	13	62	$\chi^2 = 11.7$
V	15	8	7	46.7	" 1653	1	18	94.7	$\chi^2 = 12.7$
Subtotal Lot No. 1653	51	28	23	45.1		9	48	84.2	$\chi^2 = 18.2$
Total	111	81	30	27.0		19	93	82.0	$\chi^2 = 70.6$
IA	40	Treated with Vitamin C alone.	11	27.5	Treated with hesperidino methyl chalcone alone	23	17	42.5	

killed by the arsenical in that length of time were eating well.

In the treated animals, vitamin P was administered as purified methyl chalcone of hesperidin. This substance was kindly made available by Dr. W. Biehlm, Medical Director of Abbott Laboratories. When uncertainty existed regarding whether all the arsenical was actually gotten into the vein, the mouse in question was discarded from the series.

The Control Groups. All mice whether in the control or treated groups received a dose of 27 mg of dichlorophenarsine hydrochloride per kilo of body weight. This dose was used because preliminary experiments indicated that it approximated an L. D. 85 dose of the arsenical when injected into the tail vein.

The Treated Groups. These groups received a daily subcutaneous dose of 1 mg apiece of methyl chalcone of hesperidin in saline each day for 9 days previous to and 3 days following the injection of the above dosage of dichlorophenarsine hydrochloride which also contained 2.2 millimoles of ascorbic acid per millimole of dichlorophenarsine hydrochloride. The dosage of ascorbate was chosen for convenience and because the observations of McChesney, Barlow and Klinck⁴ have indicated that it confers protection. The dose of vitamin P represented an average dose of 47.6 mg of vitamin per kg of body weight per day and was chosen arbitrarily. It is 36% greater than the dose used by Goldstein and his associates.⁷

Results. The results on the different control and treated groups are shown in Table I. It is to be noted that in each of the 5 groups of tests the treatment provided a significant protection except in the case of Group 4. It should also be noted that there is a difference in the results obtained in Groups I and II, as compared with those in Groups III, IV, and V. This may be attributed to the fact that different lots of both the vitamin and arsenical were used. Unquestionably the second lot of the arsenical was more toxic than the first lot.

Discussion. It is possible that if more mice had been used ascorbic acid alone or hesperidin methyl chalcone alone would have yielded

a statistically significant favorable result. However, a substance to counteract an acutely lethal dose of a substance must act rapidly as well as be potent. On the contrary, Horne and Scarborough found ascorbic acid to be ineffective and hesperidin to be effective in the treatment of a patient with arsenical purpura.¹ Gorrie⁸ has also reported hesperidin to be effective in the treatment of arsenical purpura.

Although we were not interested in devising a method for the assay of vitamin P, it is possible that our observations may be suffi-

⁸ Gorrie, D. R., *Lancet*, 1940, 1, 1005.

ciently consistent to be
 opment of a method for
 P active substances.

These results would it
 be advisable to provide
 abundant source of vita
 several days prior to an
 with arsenicals.

Conclusions. Ascorbic ac
 methyl chalcone when use
 therapy afforded a definite
 against the toxic effect of
 intravenously administered di
 hydrochloride.

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Antibacterial Properties of 4-Amino-2-Methyl-1-Naphthol Hydrocl

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From the Division of Bacteriology, Laboratories of the Mount Sinai Hospital, New Yo

As previously reported¹ pyridoxamine acquires antibacterial potency following irradiation at suitable wavelength and pH. Lack of activity in irradiated and non-irradiated solutions of pyridoxine, dimer and trimer of pyridoxine,[†] pyridoxal and a number of pyridine and benzene derivatives demonstrated that the presence of an amino and one or two hydroxyl substituent groups is an essential prerequisite for the development of antibacterial activity. It was tentatively assumed that the antibacterial factor may be an intermediate product of oxidation.[‡] Accordingly, it seemed to be of particular interest to determine the antibacterial potency of 4-amino-2-methyl-1-naphthol hydrochloride (i.e. water soluble derivative of vitamin K, Synkamin of

Parke, Davis) in view of the presence of an amino substituent group and the way in which the compound undergoes oxidation. The results of the studies are summarized below:

Solutions of Synkamin having the antibacterial potency were prepared, as follows:

Tenth normal HCl solution was autoclaved and cooled to 37°C prior to use. Pure Synkamin powder[§] and sodium bisulfite dissolved in concentrations of 3 and 0.5 per ml, respectively. Immediately after preparation the solution was sealed with vaseline and stored in the refrigerator.

As may be seen from Table I, Synkamin solution prepared as above proved strongly bacteriostatic against gram positive organisms in broth and gram negative bacilli in Gladstone synthetic medium. Among the few strains tested, staphylococcus, strain H and pneumococcus, type I were highly susceptible, while *Staphylococcus hemolyticus* was refractory. As may be also noted from the same

* This investigation has been supported by grants from the Commonwealth Fund and The National Vitamin Foundation.

† The author is thankful to Dr. John V. Seudi for a supply of this compound.

1 Schwartzman, G., and Fisher, A., *J. Biol. Chem.*, 1947, 167, 345.

‡ Possibly due to the formation of a lactam ring between the amino and hydroxyl groups following the elimination of a molecule of water.

§ Generously supplied by Dr. L. A. Sweet of Parke, Davis and Company.

TABLE I.
Effect of Synkamin in N/10 HCl Containing 0.05% NaHSO₃, Sealed with Vaseline.

Organism tested	Medium	Minimal amount of Synkamin	
		Complete inhibition	25 to 50% inhibition
<i>Staphylococcus</i> , Strain H	Broth	1.5-2 γ	1 γ
<i>Staphylococcus hemolyticus</i> , Strain 870	"	—	—
<i>Staphylococcus</i> , Type I	"	2.5 γ	—
Strain 42	Gladstone synthetic medium	3 γ	2 γ
" L	" " "	2.5 γ	1 γ
"	" " "	1.5 γ	0.8 γ
"	" " "	2.5 γ	1.5 γ
"	" " "	35 γ	—
<i>Staphylococcus</i> , Group I	Broth	—	—
" " "	Synthetic medium ²	0.75 γ	—
" " "	Broth	2.5-3 γ	—

— = No inhibition with 100 γ of Synkamin.

The composition of Gladstone synthetic medium and methods of determination of bacteriostatic activity and the size of inocula were the same as previously described.¹

and additional experiments not recorded in the table, gram negative bacilli (with the exception of *B. friedlander*, type A.) were readily susceptible to Synkamin. All the gram negative bacilli tested were resistant to the compound in broth, suggesting the presence of an antagonistic factor in this medium. The antagonistic effect of broth was less pronounced with meningococcus, type I, the organism being approximately only 4 times more susceptible in synthetic medium than in broth. In addition to broth, casein hydrolysate and blood serum proved antagonistic to the activity of Synkamin against *E. coli*. Various concentrations of the substances were added to Gladstone synthetic medium containing different amounts of Synkamin. The mixtures were inoculated with *E. coli*, strain 42. There was obtained 50% reversal of the antibacterial activity in the following ratios of dry weights, namely, one part of Synkamin to 125 parts of casein hydrolysate, 65 parts of mouse serum and 50 parts of rabbit serum. It remains as yet unknown whether staphylococcus would show greater susceptibility in synthetic medium than in broth. No reversal of antibacterial activity was noted following the addition of casein hydrolysate and serum to broth cultures of staphylococcus, strain H. The nature of the factor antagonizing inhibition of *E.*

coli was not yet identified.

The experiments about to be described were done in order to determine the effect of oxidation by air upon the antibacterial activity of Synkamin. The preparations were tested against *E. coli* in Gladstone synthetic medium and staphylococcus, strain H in broth. Solutions of Synkamin in N/10 HCl, in concentration of 3 mg per ml with and without vaseline seal were stored in the dark at 4°C. Twenty-four to 48 hours following preparation there occurred a 25% loss of activity in the non-sealed preparation. The titer remained stationary during the following 6 days. There was almost complete loss of activity 10 days following preparation. In the vaseline sealed preparations the titer was unchanged for the entire period of observation, i.e. 3 to 4 weeks.

Immediately upon preparation solutions containing 3 mg of Synkamin and 1 mg of sodium bisulfite in N/10 HCl gave complete inhibition of *E. coli* in concentration of 2-3 γ of Synkamin per ml of synthetic medium. On exposure to air for 4 days at 4°C there occurred a 25% loss of activity. On the other hand, addition of 2 mg of sodium bisulfite to 3 mg of Synkamin produced an immediate 40% decrease of activity which was completely restored on air exposure at 4°C for 4 days. The effect of the reducing substance upon the activity of Synkamin against staphylococcus, strain H was not as clear-cut, possibly in view of the interference of broth with the reduction.

² Frantz, I. D., *J. Bact.*, 1942, 43, 757.

a statistically significant favorable result. However, a substance to counteract an acutely lethal dose of a substance must act rapidly as well as be potent. On the contrary, Horne and Scarborough found ascorbic acid to be ineffective and hesperidin to be effective in the treatment of a patient with arsenical purpura.¹ Gorrie⁸ has also reported hesperidin to be effective in the treatment of arsenical purpura.

Although we were not interested in devising a method for the assay of vitamin P, it is possible that our observations may be suffi-

⁸ Gorrie, D. R., *Lancet*, 1940, 1, 1005.

ciently consistent to be utilized for the development of a method for the assay of vitamin P active substances.

These results would indicate that it would be advisable to provide patients with an abundant source of vitamin P and C for several days prior to and during treatment with arsenicals.

Conclusions. Ascorbic acid and hesperidin methyl chalcone when used as a combined therapy afforded a definite protection in mice against the toxic effect of a single dose of intravenously administered dichlorophenarsine hydrochloride.

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Antibacterial Properties of 4-Amino-2-Methyl-1-Naphthol Hydrochloride.*

GREGORY SHWARTZMAN.

From the Division of Bacteriology, Laboratories of the Mount Sinai Hospital, New York City.

As previously reported¹ pyridoxamine acquires antibacterial potency following irradiation at suitable wavelength and pH. Lack of activity in irradiated and non-irradiated solutions of pyridoxine, dimer and trimer of pyridoxine,[†] pyridoxal and a number of pyridine and benzene derivatives demonstrated that the presence of an amino and one or two hydroxyl substituent groups is an essential prerequisite for the development of antibacterial activity. It was tentatively assumed that the antibacterial factor may be an intermediate product of oxidation.[‡] Accordingly, it seemed to be of particular interest to determine the antibacterial potency of 4-amino-2-methyl-1-naphthol hydrochloride (i.e. water soluble derivative of vitamin K, Synkamin of

Parke, Davis) in view of the presence of an amino substituent group and the readiness with which the compound undergoes oxidation. The results of the studies are summarized below:

Solutions of Synkamin having the highest antibacterial potency were prepared as follows:

Tenth no	solution	
and cooled	prior to	
Synkamin	sodium	
dissolved	solutions of	
per ml,	immedi-	-
irradiation	was sealed	e
and stored	in refrigerator	
As a	from 7	n
solution	above	v
bacter	gram	s
in broth	negative	-
stone	medium.	v
strains	inoculated	d
pneumonia	were	e,
while	in hem	c-
	not	he

* This investigation has been supported by grants from the Commonwealth Fund and The National Vitamin Foundation.

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¹ Schwartzman, G., and Fisher, A., *J. Biol. Chem.*, 1947, 167, 345.

‡ Possibly due to the formation of a ring between the amino and hydroxyl groups following the elimination of a molecule of water.

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Organism tested	Medium	Minimal amount of Synkamin	
		Complete inhibition	25 to 50% inhibition
Staphylococcus, Strain H	Broth	1.5-2 γ	1 γ
<i>Streptococcus hemolyticus</i> , Strain 870	"	—	—
Pneumococcus, Type I	"	2.5 γ	—
<i>E. coli</i> , Strain 42	Gladstone synthetic medium	3 γ	2 γ
" " " L	" " "	2.5 γ	1 γ
<i>S. binns</i>	" " "	1.5 γ	0.8 γ
<i>S. paratyphi</i> B.	" " "	2.5 γ	1.5 γ
<i>B. friedlanderi</i> , Type A	" " "	35 γ	—
<i>E. coli</i> , Strain 42	Broth	—	—
<i>S. binns</i>	"	—	—
Meningococcus, Group I	Synthetic medium ²	0.75 γ	—
" " "	Broth	2.5-3 γ	—

— = No inhibition with 100 γ of Synkamin.

The composition of Gladstone synthetic medium and methods of determination of bacteriostatic activity and the size of inocula were the same as previously described.¹

table and additional experiments not recorded in the table, gram negative bacilli (with the exception of *B. friedlanderi*, type A.) were markedly susceptible to Synkamin. All the gram negative bacilli tested were resistant to the compound in broth, suggesting the presence of an antagonistic factor in this medium. The antagonistic effect of broth was less pronounced with meningococcus, type I, the organism being approximately only 4 times more susceptible in synthetic medium than in broth. In addition to broth, casein hydrolysate and blood serum proved antagonistic to the activity of Synkamin against *E. coli*. Various concentrations of the substances were added to Gladstone synthetic medium containing different amounts of Synkamin. The mixtures were inoculated with *E. coli*, strain 42. There was obtained 50% reversal of the antibacterial activity in the following ratios of dry weights, namely, one part of Synkamin to 125 parts of casein hydrolysate, 65 parts of mouse serum and 50 parts of rabbit serum. It remains as yet unknown whether staphylococcus would show greater susceptibility in synthetic medium than in broth. No reversal of antibacterial activity was noted following the addition of casein hydrolysate and serum to broth cultures of staphylococcus, strain H. The nature of the factor antagonizing inhibition of *E.*

coli was not yet identified.

The experiments about to be described were done in order to determine the effect of oxidation by air upon the antibacterial activity of Synkamin. The preparations were tested against *E. coli* in Gladstone synthetic medium and staphylococcus, strain H in broth. Solutions of Synkamin in N/10 HCl, in concentration of 3 mg per ml with and without vaseline seal were stored in the dark at 4°C. Twenty-four to 48 hours following preparation there occurred a 25% loss of activity in the non-sealed preparation. The titer remained stationary during the following 6 days. There was almost complete loss of activity 10 days following preparation. In the vaseline sealed preparations the titer was unchanged for the entire period of observation, i.e. 3 to 4 weeks.

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² Frantz, I. D., *J. Bact.*, 1942, 43, 757.

Additional experiments are under progress in order to investigate this relationship.

Additions of cystine, 1 mg, methionine, 3 mg, and methionine sulfoxide,[¶] 5 mg, to 3 mg of Synkamin in N/10 HCl, per ml failed to antagonize the effect of Synkamin upon *E. coli*, strain 42 and staphylococcus, strain H.

It is obvious from the above experiments that the antibacterial activity of Synkamin described is intimately related to air oxidation. Excessive oxidation brings about loss of activity. Reduction with sodium bisulfite results in reversible decrease in potency which is restored on exposure to air. It is suggestive that the antibacterial activity of Synkamin is due to an intermediate state of oxidation by air which possibly takes place immediately upon dissolving the substance. The antibacterial potency can be maintained at a constant level by timely exclusion of air from an oxidized solution or by additional oxidation of a reduced solution.

The effect of Synkamin was compared with that of 2 other water soluble derivatives of vitamin K, sodium-2-methyl-1,4-naphthoquinone diphosphate (Synkayvite, Hoffmann-La Roche) and 2-methyl-1,4-naphtho-hydroquinone-3-sodium-sulfonate (Hykinone, Abbott).[¶] The solutions were made similarly to Synkamin, with and without vaseline seal, and tested against staphylococcus, strain H in broth and *E. coli*, strain 42 in Gladstone synthetic medium. All solutions of Synkayvite were totally devoid of antibacterial activity against these organisms in concentrations 1-200 γ per ml. Immediately following preparation and following storage at 4°C, Hykinone showed no effect upon *E. coli*. After one week storage at room temperature the compound inhibited *E. coli* in the concentra-

tion of 40 γ per ml of synthetic medium. Staphylococcus, strain H, was completely inhibited by 10 γ and partially inhibited by 5 γ per ml of broth. Thus, Synkayvite possessed no activity under conditions described, while the potency of Hykinone was distinctly lower than that of Synkamin. The antibacterial effect also seems to be significantly greater with Synkamin than with vitamin K derivatives studied by previous authors,** i.e. 2-methyl-1,4-naphthoquinone and derivatives containing chloro, methyl and methoxy substituent groups.

Summary. The points of interest of the studies embodied in this note are as follows: 4-amino-2-methyl-1-naphthol hydrochloride possesses marked antibacterial activity against gram positive and gram negative organisms.

The compound is strongly effective against gram positive organisms in presence of broth, casein hydrolysate and blood serum, and against gram negative organisms in synthetic medium. The activity against the latter organisms is antagonized in broth medium. Casein hydrolysate and mouse and rabbit sera antagonize the antibacterial activity of Synkamin against *E. coli* in a significantly lesser degree than broth, namely, in dry weight ratios of 125:1, 65:1, 50:1, respectively.

The presence of an amino substituent group and partial oxidation of the substance under carefully controlled conditions play an important role in the antibacterial activity described.

The author is indebted to Miss Alice Fisher for capable and accurate assistance.

** No detailed comparison can be presented in this brief communication. Reports of previous investigations are listed under References.³

³ Fosdick, L. S., Faneher, O. E., and Calandra, I., *Science*, 1942, 96, 45; Armstrong, W. D., Spink, W. W., and Kahnke, I., *Proc. Soc. Exp. Biol. and Med.*, 1943, 53, 230; Wooley, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 225; Colwell, A., and McCall, M., *J. Bact.*, 1946, 51, 659; and Hoffmann-Ostenhof, O., *Science*, 1947, 107, 549.

[¶] Kindly made available by Dr. Joseph Seifter of Wyeth Institute.

[¶] Thanks are due to Dr. Elmer L. Sevringhaus of Hoffmann-LaRoche, Inc., for a supply of Synkayvite and to Dr. Arnold E. Osterberg of Abbott Laboratories for a supply of Hykinone.

Protection of *Escherichia coli* against Bacteriophage with Citrus Pectin.

F. D. MAURER* AND D. W. WOOLLEY.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Since the discovery in this laboratory^{1,2} that apple pectin and certain other polysaccharides inhibit the hemagglutinating activity and the multiplication of influenza A virus it became desirable to determine whether other viruses as well could be combatted with such polysaccharides. A suitable bacteriophage acting on *Escherichia coli* suggested itself as a useful system for this purpose, not only because of the facility with which various aspects of the virus-host relationship might be studied, but also because circumstantial evidence has pointed to the conclusion that the point of viral attack on the bacterium is carbohydrate in nature.³ This latter point was attractive because the discovery of the effect of apple pectin in inhibiting influenza virus was based on belief in the polysaccharide nature of the cell receptor of this virus.^{1,2} Just as in the case of influenza A virus, so with bacteriophage the host cells might be protected by supplying an antagonistic polysaccharide to compete with the receptor substance. For these reasons pectins were examined for ability to protect the bacterium from the phage, and it was found that citrus pectin was capable of doing this.

To demonstrate this phenomenon, the T₂ phage and its susceptible host strain of *Escherichia coli* were best suited.¹ Inocula for the tests were prepared by incubating the strain of *E. coli* in a synthetic medium⁴ for 2.5 hours at 37°C. At this time growth was

just plainly visible. 0.5 cc of this fresh young culture was used to seed each tube of the synthetic basal medium of Hook *et al.*⁵ which was used in all of the tests, unless otherwise noted. The inoculum of T₂ bacteriophage was prepared by growing the virus in the susceptible strain of bacteria cultivated in Todd-Hewitt broth⁶ for 20 hours at 37°C. The lysate was filtered through a Seitz pad to provide cell-free material and the filtrate was titered by the plaque count method.⁷ Suitable dilutions were then used in the tests. The same phage filtrate was used throughout this investigation, and was maintained potent by storage at 4°C. Solutions of citrus pectin were prepared exactly as described previously,² and no other manner of obtaining solutions was found satisfactory.

When the synthetic basal medium of Hook *et al.* fortified with 100 mg of citrus pectin

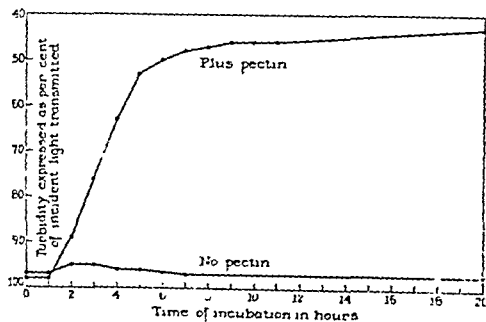


FIG. 1.
Effect of pectin on the growth curves of *E. coli* in the presence of T₂ bacteriophage.

* Major, Veterinary Corps, U. S. Army.

¹ Woolley, D. W., and Green, R. H., *J. Bact.*, 1947, **54**, 63.

² Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **80**, 55.

³ Burnet, F. M., Koegh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med. Sci.*, 1937, **15**, 227.

† We wish to thank Dr. S. E. Luria for transplants of these organisms.

⁴ Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

⁵ Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

⁶ Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.

⁷ Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 267.

TABLE I.
Influence of Citrus Pectin on Multiplication of *E. coli* and T_2 Bacteriophage. Counts Made After 20 Hours Incubation at 37°C.

Additions to basal medium	<i>E. coli</i> cells per cc	Phage particles per cc
Bacteria	1×10^9	
" + pectin	1×10^9	
" + phage	0	11×10^9
" + pectin + phage	2×10^9	5×10^9

per 6 cc was inoculated with 10^8 cells and then with the 10^9 phage particles, and the mixture was incubated at 37° overnight, heavy growth of the bacteria occurred. In contrast, when no pectin was added, no bacterial growth was visible. In the absence of pectin the virus had acted in its usual fashion and had lysed the host. Furthermore, the citrus pectin was not injurious to the growth of the bacteria in the absence of phage, because multiplication of coli in tubes of medium plus pectin was equal to that in the basal medium without pectin. Growth curves of *E. coli* in the presence of the T_2 phage, with and without citrus pectin are shown in Fig. 1. Quantitative data on the numbers of viable cells in experiments such as these will be found in Table I.

The concentration of citrus pectin needed to effect this protection of the bacterial cells was determined by adding graded amounts of the substance to the basal medium which was subsequently inoculated with *E. coli* and with T_2 phage, and incubated for 20 hours. Sixty mg of citrus pectin per 6 cc of culture was the smallest quantity which would allow good growth of the bacteria.

A number of polysaccharides were tested for ability to protect against the phage, and citrus pectin was found to be the most effective. Apple pectin at 125 mg per 6 cc would not allow growth of the bacteria in the presence of the virus when the incubation was for 20 hours, but in a test of 40 hours' duration this substance was almost as effective as citrus pectin. Gum acacia (125 mg per 6 cc†) or starch (125 mg per 6 cc) were inactive. This order of potency was not the same as that for these substances acting against influenza virus. In that instance Green and

Woolley² found that apple pectin was more active than citrus pectin in causing inhibition of multiplication of the virus, and that gum acacia had some potency. All 3 substances, but not starch, were inhibitors of hemagglutination caused by the virus.

The power of citrus pectin to enable *E. coli* to grow luxuriantly in the presence of its bacteriophage is the basic observation in this work. The experiments which are now to be outlined were performed in an effort to learn how this action was accomplished.

It was first necessary to ascertain whether the action of citrus pectin was attributable to the increased viscosity produced by it in the medium. Work of earlier investigators has led to the conclusion⁵ that substances which made the medium highly viscous protected bacterial cells from destruction by phage. This old opinion proved not to be entirely correct, because in the present work it was found that viscosity and protective action against the virus were not always correlated. Potent protective substances with very low viscosity, as well as inactive materials of high viscosity were both found. As D'Herelle has stated many viscous substances exert protective action against lysis, but this correlation proved to be illusory because non-viscid material which was still quite active was found. Thus, a viscous material such as gelatin at 60 mg per cc of the culture fluid allowed good growth of *E. coli* in the presence of phage, and in this respect resembled citrus pectin. Both media were viscid (time of flow of the gelatin fortified basal medium 524 seconds in an Ostwald viscosimeter at 37°C and of the pectin containing medium 259 seconds). However, when the gelatin was digested with crystalline trypsin overnight,

† Subsequent experiments showed that 240 mg of acacia per 6 cc did exert some effect.

⁵ D'Herelle, F., *The Bacteriophage and Its Behavior*, Williams and Wilkins Co., 1926.

TABLE II.

Influence of Citrus Pectin on Survival of Cells in Presence of Bacteriophage as Shown by Plate Counts Made 15 Minutes After Mixing the Participants.

Additions to basal medium	Numbers of organisms after 15 min.	
	$10^2 \times E. coli$ cells per cc	$10^8 \times$ phage particles per cc
Phage		3
Bacteria	4,000,000	
" + pectin	5,000,000	
" + phage	3	1
" + pectin + phage	3,000,000	3

and the digest was dialyzed against running water, the nondialyzable portion retained full (and even slightly enhanced) powers of protection against lysis by the phage. Digestion so reduced the viscosity that basal medium containing the preparation at a level equivalent to 60 mg of gelatin per cc was scarcely more viscous than the basal medium without any additions (time of flow 99 seconds compared to 84 seconds). Possibly the polysaccharide portion of gelatin which remained after the proteolysis, was responsible for the protective effect against the phage. The lack of correlation of viscosity and protection from lysis may be demonstrated in the reverse manner. Thus, the basal medium fortified with Tween 80 (125 mg per cc) at pH 5.7, was more viscous than that containing an effective concentration of citrus pectin. Nevertheless, the Tween did not prevent lysis of the bacteria by the virus. Therefore, the protection afforded by citrus pectin was not due solely to the fact that it increased the viscosity of the medium.

The effect of the pectin was not to destroy the phage, because no virucidal power could be demonstrated. Thus, when 10^{10} phage particles were added to citrus pectin solution (20 mg per cc), and the mixture incubated for 30 minutes, diluted and assayed by the plaque method, 10^{10} particles were found.

The data of Table I show that while citrus pectin allowed the bacteria to grow in the presence of the phage, it did not prevent multiplication of the virus. Thus, nearly as many virus particles were found in 20-hour cultures containing abundant viable bacterial cells supported by pectin, as in the control cultures without pectin where few cells survived. This is exactly the situation which

obtains with lysogenic strains⁹ in which one finds growth of the host strain, and no lysis, concomitant with multiplication of the phage. Therefore, the addition of citrus pectin to the system of *E. coli* and T₂ phage has reproduced the phenomenon of lysogenic strains. This change was dependent on the continued presence of the pectin.

Citrus pectin did not prevent the attachment of the virus to the host cell, but it did protect the cell from lysis, *i.e.*, destruction. This protection from lysis may be seen from the results of the following experiments. Two tubes of basal medium, one containing pectin (100 mg per 6 cc), and the other none, were inoculated with approximately 10^8 young cells and 10^8 phage particles and the mixtures were incubated at 37°C for 15 minutes. During this time no multiplication of the bacteria would be expected. Dilutions were made, and in these the numbers of viable cells and of phage particles were determined by the usual plate counts. The results of a typical run are shown in Table II, where it can be seen that in the tube without pectin most of the viable cells had become infected and subsequently disappeared through lysis while in the tube with pectin no significant decrease had occurred.

The fact that pectin did not prevent attachment of the phage to the cells was shown in the following way. The experiment immediately preceding was repeated, except that after the 15-minute incubation period the cells were collected by centrifugation and washed 4 times with a solution of citrus pectin (20 mg per cc). Dilutions were then made in water, and the numbers of viable cells and of phage

⁹ Northrop, J. H., *J. Gen. Physiol.*, 1939, 23, 59.

particles were determined. The object of the pectin washing was to remove any virus particles which were not attached to cells. The results showed that in the tube which contained pectin when the host and parasite were mixed, 2×10^8 phage particles, the number actually introduced as inoculum, still remained. These must therefore have been fixed to the cells in a manner firm enough not to be dislodged by washing. The results were similar when water or physiological saline were used as wash. However, the pectin solution was used as wash so that excess phage could be removed without danger of subsequent infection of the cells. If water had been used, the pectin in the culture would have been diluted and thus rendered impotent before the excess of virus had been eliminated. Infection of the cells subsequent to the main experiment might thus have occurred.

From the above experiments it seemed probable that the pectin formed a protective layer around the bacterial cells. This layer did not prevent the attachment of the virus to the host; but it did preclude destruction of the cells. Furthermore, it did not prevent the multiplication of the virus. Moreover, this protective influence of the pectin remained with the cell during dilution of the culture and subsequent plating on agar. This fact is well illustrated by the data in line 5 of Table II.

An apparently therapeutic effect of citrus pectin on cells previously infected with the phage was observed, but this was shown to be spurious, and to have resulted from a very few uninfected cells among the infected ones. Special means were required to detect these uninfected cells, because by the ordinary plating methods none could be found. Thus, a suspension of 10^8 fresh young cells was mixed with 10^9 phage particles and the whole was incubated for 15 minutes. The cells were then collected by centrifugation and washed to remove excess virus. They were then used as inoculum for two tubes, one containing basal medium and the other basal medium plus citrus pectin. The tubes were incubated at 37° for 20 hours, and it was then found that the one with pectin contained luxuriant growth, while the one without had

none. Plate counts had been made on dilutions of the inoculum of infected cells, and no living bacteria had been detected. However, when the counting was done on plates made from pectin-containing basal medium (solidified with agar) a few (43) viable, and therefore presumably uninfected cells were found. One may conclude that the presence of the pectin in the plating medium protected those uninfected cells from attack by the large amounts of virus liberated from their infected neighbors, and thus allowed detection. The probable explanation of the phenomenon observed in the tubes now seemed clear. The few cells which escaped infection during the period of exposure to phage were thereafter protected from the phage by the addition of pectin and proceeded to multiply.

The protection of bacterial cells from destruction by bacteriophage was not an isolated observation confined to one strain of the organisms employed. The same relationships were found with *E. coli* strain No. 8677 of the American Type Culture Collection in combination with either the T_2 phage, or with phage strain 8677 of the A.T.C.C. This bacterium, however, was less well adapted for the experiment than was the Luria strain, because when incubation of it with the phage was continued for 20 hours or longer, overgrowth of the previously lysed culture occurred. The effect of pectin, therefore, could be observed for a much shorter time.

One additional fact was, however, established with the A.T.C.C. organisms, namely, that the amount of pectin needed for protection was practically independent of the number of phage particles introduced. The amount of phage was varied over a 100-fold range without altering the influence of a minimal effective amount of pectin.

The discovery of the inhibiting action of apple pectin and certain other polysaccharides on influenza virus^{1,2} arose from a working hypothesis which postulated a specific polysaccharide in the host cell which the virus attacked. This receptor substance was postulated to compete with pectin for the attention of the virus because of structural analogy of the two. Woolley (in press) has pursued this working hypothesis further, and has purified

from erythrocytes of species susceptible to the virus, a polysaccharidelike substance which does compete with apple pectin for the virus acting as a hemagglutinin. Therefore, a search for a substance in *E. coli* which might antagonize the protective action of citrus pectin on this species seemed advisable. A number of attempts to demonstrate such a substance have been made, but none has succeeded. The testing procedure employed for this was to add the various samples to the basal medium plus citrus pectin (100 mg per 6 cc) and then to inoculate with 10^8 young cells and with 10^9 phage particles. Incubation of the mixtures for 20 hours should reveal an active preparation by failure of the bacteria to grow. This should follow logically because if the pectin were competing with a cell receptor, increasing the total concentration of the latter in the system should nullify the action of the pectin. The concentration of this receptor in the system is fixed by the number of bacteria, but it might be increased by adding extracts of the substance from the cells. Extracts were prepared from 20-hour cultures of *E. coli* by treating the cells

with weak alkali, by grinding them with sand, by heating to 70° or 100° , by ultraviolet light irradiation, and by plasmolysis with ether or with chloroform. None was found able to overcome the antiviral action of citrus pectin. Needless to say, the influenza virus receptor substance extracted from human erythrocytes likewise was ineffective.

Summary. Citrus pectin was found to allow growth of *E. coli* in the presence of an excess of bacteriophage. The pectin was non-toxic to the bacteria, and was not virucidal. The action of the pectin was found not to be the prevention of attachment of the virus to the cell, and indeed, it did not inhibit the multiplication of the phage appreciably. It did, however, protect the cells from lysis, and hence in its presence a phenomenon similar to that seen with lysogenic strains was observed. Gum acacia and starch, representatives of other classes of polysaccharides, were ineffective. No substance could be demonstrated in the cells which was antagonistic to citrus pectin in the way that an influenza virus substrate in erythrocytes was antagonistic to apple pectin.

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Effect of Thyro-Parathyroidectomy upon the Blood and Plasma Volumes of the Rat.

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There have been few studies of blood and plasma volumes in myxedema or experimental hypothyroidism, none on the rat. Thompson¹ in an early study found the plasma volume in

myxedematous patients to be 30% below normal, and also found that the values returned to normal upon treatment with thyroid extract. Holböll² likewise found the blood and plasma volume reduced in myxedema. In the most recent study, Gibson and Harris³ studied 7 myxedematous patients. They found that

* The authors gratefully acknowledge the technical assistance of W. Lew and W. Wong. Hemoglobin solution was provided through the courtesy of Dr. Robert B. Pennell, Sharp & Dohme, Inc., Philadelphia, Pa. This study was supported by a grant from the U. S. Public Health Service. Dr. Lippman is now at the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles.

¹ Thompson, W. O., *J. Clin. Invest.*, 1926, **2**, 477.

² Holböll, S. A., *Acta med. Scandinav.*, 1930, **73**, 538.

³ Gibson, J. G., 2nd, and Harris, A. W., *J. Clin. Invest.*, 1939, **18**, 59.

the blood volume was only 15% below normal, and attributed the lower results of earlier authors to errors in the method of blood volume determination used.

Methods. This study utilized 16 male rats, ranging in weight from 150 to 180 g at the start. Thyro-parathyroidectomy was performed, under ether anesthesia, by blunt dissection and cauterization of the thyroid bed. Following this, an interval of 3 weeks elapsed, during which the rats were undisturbed and were fed upon stock diet. At the end of 3 weeks, the rats had failed to gain weight in accordance with the normal growth curve. Blood and plasma volume determinations were performed according to the hemoglobin dye method previously described.⁴ Gross post-

mortem examination showed no regeneration of thyroid tissue at the site of operation. The heart weights were diminished in accordance with previous experience upon thyro-parathyroidectomized rats.⁵

Results. Plasma volumes were obtained in 16 rats, and blood volumes were obtained in 14 of these. There was a mean reduction of plasma volume, erythrocyte volume and total blood volume of approximately 10%, when compared with normal standards for rats of the same weight, as previously established.⁴ (Table I).

⁴ Lippman, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 188.

⁵ Addis, T., Karnofsky, D., Lew, W., and Poo, L. J., *J. Biol. Chem.*, 1938, **124**, 33.

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Protective Action of Dietary Cholesterol in Experimental Thyrotoxicosis.*

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From the Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles, Calif.

Experiments were made to investigate further the well known effects of hyperthyroidism on cholesterol metabolism.¹ An unexpected result of these studies was the observation that a reverse relationship also existed, *i.e.* that high dietary cholesterol modified the toxic effects of high doses of thyroid hormone, in particular, as far as survival time and body weight changes were concerned. These findings are reported in the present communication.

Experimental Procedure. Normal rats were fed *ad libitum* this laboratory's stock diet, slightly modified to contain more yeast, in order to assure an adequate supply of the vitamin B complex.[†] In addition, thyroid powder was mixed with the diet at concentra-

tions corresponding to 0.4-0.8% thyroid U.S.P. One group of rats (T) received the high thyroid diet as described, a second group (T + C) was fed an identical diet, except that it contained in addition, 1% cholesterol and 0.5% bile salt. The cholesterol was dissolved in cottonseed oil, replacing a corresponding amount of the oil in the diet. The bile salt was added to increase absorption of cholesterol.² The rats were about 9 months (Experiment 1) and 38 days (Experiment 2) old respectively at onset of the feeding periods. Litter mates were distributed evenly between the two groups. Body weights were measured once weekly, and the survival time

* Supported by a grant from the Life Insurance Medical Research Fund.

¹ Peters, J. P., and van Slyke, D. D., *Quant. Clinical Chem., Interpretations I*, 2nd Edition, 1946, 497.

[†] Modified stock diet: 325 g whole wheat flour; 327 g ground oats; 100 g skimmed milk powder; 30 g alfalfa; 95 g yeast, Anheuser-Busch strain G; 80 g cottonseed oil; 20 g fortified cottonseed oil, containing 2500 I.U. vitamin A/g and 400 I.U. vitamin D₂/g; 5 g NaCl; 5 g CaCO₃.

² Schoenheimer, R., *Biochem. Z.*, 1924, **147**, 258.

TABLE I.
Effect of High Dietary Cholesterol on Survival Time of Rats Fed Toxic Doses of Thyroid Hormone.

Exp. No.	Diet	No. of rats	Age at onset	Mean survival in days	Standard error of mean*	$\frac{D_M^\dagger}{S_{D_M}}$
1	T	6	9 mos.	23	± 4.3	13.4
	T + C	6	9 "	55+	0	
2	T	14	38 days	86	± 3.9	3.5
	T + C	7	38 "	111+	± 5.4	

* The standard error of the mean calculated from the formula $S_M = \frac{\sqrt{\sum d^2}}{n(n-1)}$ where "d" is the deviation from the mean, and "n" is the number of observations.

† Ratio, difference of means (D_M) to standard error of difference (S_{D_M}); results are considered significant when this ratio > 3 .

$$S_{D_M} = \sqrt{\frac{\sum d_1^2 + \sum d_2^2}{(n_1 - 1)(n_2 - 1)}} : \frac{n_1 n_2}{n_1 + n_2}$$

was noted. In the second experiment, the food intake was also measured, the animals being kept in single cages with screen bottoms.

Results. The experimental results are summarized in Table I and Fig. 1. In the first experiment, in which older rats and a desiccated hog thyroid preparation containing 0.65% total I and 0.31% thyroxine were used, the high dietary cholesterol had striking effects, both appreciably prolonging the length of survival time and protecting against the loss in body weight. All rats of group T died within 5-36 days, the average survival time being 23 days. In group T + C, all animals were alive at 55 days, at which time the animals were killed for autopsy. Fig. 1 indicates that the excessive weight loss caused by thyroid hormone (group T) also was prevented or diminished by the addition of cholesterol + bile salt (group T + C).

In Experiment 2 in which young rats and a different hormone preparation† were used, the same trend was observed, but the protective action of cholesterol was less marked. The difference in the mean survival times between the groups T and T + C was still significant. The body weight results were equivocal, however; only in the case of the male rats was a similar trend observed as in

Experiment 1.

The possibility was considered that the animals on the high cholesterol diet (T + C) ate less due to the presence of bitter-tasting bile salt in their food. In this case the amount of thyroid hormone consumed by this group would have been correspondingly smaller. In order to investigate this possibility, the food consumption was measured in Experiment 2. The results indicate that the animals ate practically the same amount of food in both groups. The average intake per day was 17 g in group T, and 16 g in group T + C. It is highly improbable that such an insignificant difference in the amount of food and thyroid hormone consumed was responsible for the marked differences in survival time and body weight observed. The latter differences have to be attributed, therefore, to an action of the cholesterol protecting from the toxic effects of thyroid hormone.

This conclusion was confirmed by experiments made recently in collaboration with Dr. B. Ershoff.³ In those experiments, conditions were essentially the same,[§] except that bile salt was entirely omitted from the diet. It can be assumed that under these con-

³ Ershoff, B. H., and Marx, W., to be published.

[§] A different strain of rats and a different stock diet were used; the cholesterol was mixed with the diet as a dry powder (for group T + C).

† Thyroid powder, U.S.P. Armour.

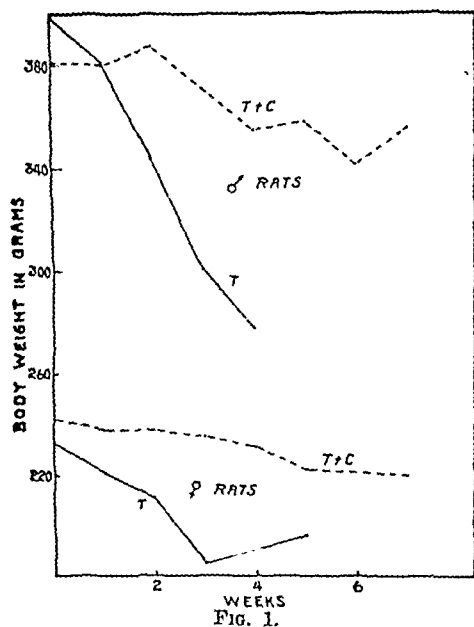


FIG. 1.
Effect of dietary cholesterol on the body weight of rats fed toxic doses of thyroid hormone. T = thyroid alone; T + C = thyroid + cholesterol.

ditions both groups T and T + C consumed the same amount of diet, and therefore, of thyroid hormone. It is interesting that, in this experiment, the survival time was again significantly prolonged as a consequence of the high dietary cholesterol, in spite of the fact that the absorption of the latter was very likely smaller than in the experiments discussed above.

Discussion. The authors are not aware of any report in the literature referring to experimental observations on a protective effect of

high dietary cholesterol in case of thyrotoxicosis. The findings are in agreement, however, with a hypothesis recently proposed by Hoffmann and Hoffmann.⁴ According to this theory, thyroid hormone stimulates the enzymatic breakdown of lecithin to lysolecithin. Lysolecithin in turn, is believed to affect the central nervous system, to influence cell permeability, and to exert, at higher concentrations, "toxic effects in various organs." These are the effects which cholesterol is supposed to counteract, or to neutralize. It might be added, in connection with this concept, that an antagonism between phospholipids and cholesterol was postulated by various investigators, and many instances can be quoted where such an antagonism was demonstrated experimentally.⁵

Summary. High dietary cholesterol prolonged significantly the survival time of rats fed toxic doses of thyroid hormone; in some, but not in all groups the animals' weight loss was also reduced.

We are very grateful to Mr. William C. Werkheiser of this department for determinations of iodine and thyroxine on a sample of desiccated thyroid, and to Dr. Frederic Fenger of the Armour Laboratories, and to Mr. Noble F. Payton of the Suburban Chemical Co., Chicago, for the cholesterol used in this work.

⁴ Hoffmann, F., and Hoffmann, E. J. de, *Public. del Institut. Fisiol., Universidad de Chile*, 1943; quoted by Foldes, F. F., and Murphy, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 218.

⁵ Bills, C. E., *Physiol. Rev.*, 1935, **15**, 1.

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$$S_{D_M} = \sqrt{\frac{\sum d_1^2 + \sum d_2^2}{(n_1 - 1)(n_2 - 1)}} : \frac{n_1 n_2}{n_1 + n_2}$$

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[‡] Ershoff, B. H., and Marx, W., to be published.

[§] A different strain of rats and a different stock diet were used; the cholesterol was mixed with the diet as a dry powder (for group T + C).

[‡] Thyroid powder, U.S.P. Armour.

TABLE I.
Chronic Toxicity of SN 13592—Test 1-U.

Group	No. of rats	Compound	Fraction of lethal dose daily	Total mg/kg (11 days)	No. of animals surviving	Avg wt gain (g/11 days)
A	6	Control	—	—	6	49.8
B	6	Quinine	1/16	308	5	45.4
C	6	"	1/8	616	6	50.0
D	6	"	3/16	924	6	49.0
E	5	SN 13592	1/16	3432	4	42.5
F	6	"	1/8	6875	6	60.3
G	6	"	3/16	10307	5	40.0

TABLE II.
Chronic Toxicity of SN 13592—Test 1-A. Five chicks in each group.

Group	Compound	Dose mg/kg/day	No. of chicks surviving	Avg wt change (g/4 days)
A	Vehicle	—	5	+22.4
B	Quinine	350	4	+16.2
C	"	200	5	+25.8
D	"	100	5	+25.8
E	"	50	5	+28.0
F	SN 13592	4000	4	— 1.2
G	"	2000	4	+11.0
H	"	500	5	+28.8
I	"	100	5	+20.8

gain with dosage, as shown in Table II.

Over a period of almost 6 weeks, one Rhesus monkey received a total of 3.6 g orally, in doses beginning at 10 mg twice a day, and progressively doubling each week. During this time no digestive or physiological disturbances were observed other than a hematological change. The blood picture altered markedly with the appearance of a decrease in red blood cells and a concomitant decrease in hemoglobin. Fig. 1 describes the rather rapid induction of this anemia, and the almost total recovery during the 39 days after

cessation of SN 13592 administration. An attempt to induce the same type of anemia in rabbits and rats was unsuccessful.

Local Effects. Ten per cent solutions of calcium pantothenate have been found to have no irritating effect on the conjunctival mucosa of the rabbit.⁶ In the present study no gross or microscopic pathology was observed in the vaginal tissues of animals in which SN 13592 was applied to the vaginal mucosa in saline. In 20 treatments over a 10-day period 4 rats received a total of 20 mg each, 2 guinea pigs a total of 50 mg each, and 2 rabbits a total of 200 mg each.

Systemic Effects. The respiratory effect observed upon oral and intravenous administration of SN 13592 led to preliminary studies of its effect on respiration, circulation and smooth musculature. Intravenous administration evoked inconsistent variations in blood pressure as determined by carotid cannulation and a mercury manometer. Doses as high as 50 mg/kg in the cat induced only relatively minor changes in blood pressure. The heart rate showed similar minor variations in this range of drug administration.

The effect of intravenous SN 13592 on respiration is almost immediate, although of

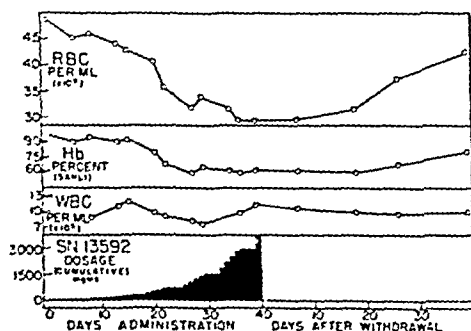


FIG. 1.

Hematological changes upon continued administration of SN 13592 and after its withdrawal.

Toxicity and Pharmacology of SN 13592.* An Analogue of Phenyl Pantothenone.

HERON O. SINGER, NATHAN MILLMAN, AND MARY RICE BOSWORTH.

From the Ortho Research Foundation, Raritan, N.J.

The oral antimalarial activity of phenyl pantothenone¹ led to the preparation of compounds which might be considered as thio analogues.² One of these was SN 13592,³ (-)- α,γ -dihydroxy- β,β -dimethyl-N-(2-(phenyl-mercapto)-ethyl)-butyramide. This compound was examined for trichomonocidal activity.⁴ Since high *in vitro* activity was demonstrated, parallel studies of toxicity and systemic effects of this substance were conducted, as representative of the type of phenomena which might be shown by a series of similar phenyl pantothenone analogues. The toxicity tests, for the most part, were based on those described in the *Survey of Antimalarial Drugs*,³ with minor modifications. The tests are referred to by the designations given them in that publication.

Acute toxicity. Pantothenic acid has an extremely low oral toxicity for the rat. The lethal dose is greater than 10 g/kg.⁵ SN 13592 is characterized by a similar low toxicity, being of the order of 5 g/kg. The administration of larger doses by means of the stomach tube was difficult because of the low solubility of the compound. Death at toxic levels of calcium pantothenate is the result of respiratory failure, although lower doses do not seem to affect the respiratory system.⁶ With SN 13592, all doses above 500 mg/kg were attended by marked respiratory difficulty, which usually disappeared within 1-2 hours in surviving

animals. This respiratory effect was the subject of further study, as described under "systemic effects."

While acute intravenous toxicity was not investigated, it was noted that in one case a dog was infused with a total of 2.6 g (397 mg/kg) over a period of 5 hours without fatal result.

Chronic toxicity. Tests were performed to determine the chronic toxicity for several species of animals. An 11-day rat test was conducted according to the procedure of Test 1-U,³ with the deviations in weight gains between experimental and control animals as the criterion. During the experimental period, groups of rats receiving 1/16, 1/8, or 3/16 of the lethal dose daily averaged a weight gain similar to that of control rats fed the vehicle alone, and also similar to that of control rats fed the same fraction of the lethal dose of a reference compound, quinine. As shown in Table I, there were a few deaths, but it is doubtful whether these were attributable to the drugs since they also occur with the reference drug.

Histological examination of major organs of these rats, including the intestinal tract, revealed no significant pathological changes.

A 7-day mouse test (Test 1-B) gave similar results as far as weight gains were concerned. Groups at 45, 90, and 270 mg/kg/day exhibited average weight gains comparable to the control animals, but mortality was much higher at daily doses greater than 270 mg/kg.

For studies with the chick Test 1-A was used. The maximum tolerated dose (that daily dose at which the final weight is approximately equal to the starting weight) was determined to be 4000 mg/kg/day for the 4 days of the test. However, at 500 mg/kg/day the chicks gained as much weight as the controls, and as much as the group fed 50/kg/day of the reference drug quinine. It is interesting to note the variation in weight

* The Survey Number, SN 13592, designates this compound in the *Survey of Antimalarial Drugs*.

1 Woolley, D. W., and Collyer, M. L., *J. Biol. Chem.*, 1945, **159**, 263.

2 Senear, A. E., Rapport, M. M., and Koepfli, J. B., *J. Biol. Chem.*, 1947, **167**, 229.

3 Wiselogle, F. Y., *A Survey of Antimalarial Drugs*, Edwards, Ann Arbor, 1946.

4 Johnson, G., and Kupferberg, A. B., in press.

5 Molitor, H., *Fed. Proc.*, 1942, **1**, 309.

6 Unna, K., and Greslin, G., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 311.

TABLE I.
Effect of Analogues of Pantothenic Acid upon Strain No. 2 *Trichomonas vaginalis*.

No.	Compound	Observations on 9th day	
		Lethal at one part in: × 1000	Complete inhibition at one part in: × 1000
1.	SN 13593 (+)- α - γ -Dihydroxy- β , β -dimethyl-N-(2-(phenylsulfinyl)-ethyl)-butyramide	8	16
2.	d(+)-(pantoyltauryl)-p-anisidide	10	20
3.	SN 12610 N-(2-Benzylethyl)- α - γ -dihydroxy- β , β -dimethyl-butylamide. Prepared from natural (—) lactone	400	500
4.	SN 13592 (+)- α - γ -Dihydroxy- β , β -dimethyl-N-(2-(phenylmercapto)-ethyl)-butyramide	750	—
5.	" Decolorized by norite	800	—
6.	" Crystalline fraction	400	
7.	" Fraction boiling up to 55°C at 10 ⁻⁴ mm Hg	400	
8.	" Fraction boiling 55-155°C at 10 ⁻⁴ mm Hg	400	
9.	" Fraction boiling 155-190°C at 10 ⁻⁴ mm Hg	400	

Compounds 1 and 4 were received from Dr. J. B. Koepfli, California Institute of Technology. No. 2 was supplied by Dr. J. P. English, American Cyanamide Co. No. 3 was supplied by Dr. R. E. Lutz, University of Virginia. Nos. 5, 6, 7, 8, and 9 were prepared by Dr. Wm. Orshnik and Mr. R. A. Mallory in our own Division of Organic Chemistry.

TABLE II.
Inhibition of Eight Strains of *T. vaginalis* by SN 13592. (Decolorized by Norite.)

Strain No.	Effective dilutions one part in: × 100,000	Not effective at one part in: × 100,000
1	10	11.2
2	8	—
3	14	16
4	7	9
5	9	10
6	6	7
7	2	3
9	9	10

Strain No. 1 was isolated in 1939.³

Strains No. 2-No. 7 were isolated in 1945.²

Strain No. 9 was isolated in 1947 from a case of acute vaginal trichomoniasis with the assistance of C. E. Folsome, M.D.

observed microscopically after 3, 6, and 9 days.

The data presented in Table I summarize the results obtained with a series of compounds using strain No. 2 as the test organism. Table II shows the comparative sensitivity of 8 strains of *T. vaginalis*.

The data in Table II reveal a sevenfold variation in sensitivity to SN 13592, ranging from 1:200,000 to 1:1,400,000.

The following compounds were found to

³ Trussell, R. E., and Plass, E. D., *Am. J. Obst. and Gynec.*, 1940, 40, 883.

have little or no activity at a concentration of 1:10,000:

1. dl-N-Pantoylisoamylamine
2. dl-N-Pantoyl-N-butylamine
3. dl-N-Pantoylathanolamine
4. d(+)-Pantoyltauryl-p-nitroanilide
5. d(+)-Pantoyltauryl-p-benzylamide
6. 2-(d(+)-Pantoyltauryl)-amino-5-Chloro-pyridine
7. d(+)-Pantoyltauryl-p-Carboxyanilide
8. dl-N⁴-Pantoyltauryl-Sulfanilamide

Compounds 1, 2, and 3 were supplied by Dr. Wm. Shrive, University of Texas. Compounds 4 through 8 were received from Dr. J. P. English, American Cyanamide Company.

Table III shows that the sensitivity of *T. foetus* to the pantothenate analogue is in the same range as that of *T. vaginalis*. The single strain of *T. gallinae* appears to be slightly more sensitive than most strains of *T. vaginalis*. As noted in a previous publication¹ these other trichomonad species grow

TABLE III.
Effect of Norite Treated SN 13592 on *T. foetus* and *T. gallinae*.

Organism	No. surviving cells at one part in × 10,000
<i>T. foetus</i> 3P	30
" BR	40
" AN	50
<i>T. gallinae</i>	100

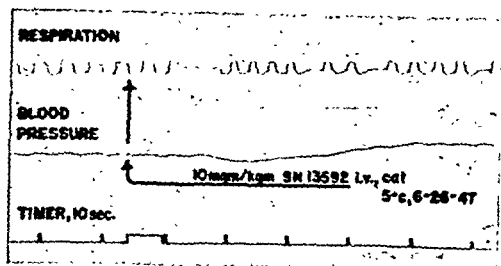


FIG. 2.

Blood pressure and respiratory system effects of SN 13592.

short duration. Respiration became shallower and erratic in the cat after intravenous injection of doses greater than 10 mg/kg (Fig. 2). In some cases it ceased entirely for an interval of a few seconds. Control injections of the vehicle alone failed to produce this response.

It was also found that the compound had a small effect on smooth musculature. *In vitro* intestinal strip preparations were slightly stimulated by 1:2000 dilutions. Ten mg/kg of SN 13592 administered intravenously in the cat enhanced uterine contractility.

Summary. SN 13592 is characterized by an extremely low acute and chronic oral toxicity in several species. Local irritation effects are absent even upon repeated administration. Continued oral feeding of the drug induces an anemia which gradually disappears after the drug is withdrawn. Although there were no consistent effects on blood pressure and heart rate, a respiratory effect was noted. Smooth muscle was generally stimulated by this compound.

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Chemotherapy of Bacteria-Free *Trichomonas vaginalis*. III. Action of Analogues of Pantothenic Acid.*

GARTH JOHNSON AND ALFRED B. KUPFERBERG.

From the Division of Microbiology, Ortho Research Foundation, Raritan, N.J.

The recent demonstration by Kupferberg *et al.*¹ that *Trichomonas vaginalis* requires pantothenic acid motivated this study of the inhibitory effects of pantothenate analogues.

Materials and Methods. The effect of a variety of compounds as growth inhibitors was first explored using strain No. 2 of bacteria-free *Trichomonas vaginalis*.² Refined studies were later run using 7 additional pure cultures of *T. vaginalis*. In addition 3

strains of *T. foetus*[†] and one strain of *T. gallinae* were explored using a single highly active compound.

One ml of an appropriate stock dilution of each compound was added to 9 ml of Simplified Trypticase-Serum medium¹ containing 3.2 µg of added calcium pantothenate per 10 ml of final medium. The solvent used was either water or 95% ethanol. In no case where alcohol was used as a solvent was more than 0.1 ml of alcohol introduced into 10 ml of culture medium. In such instances a control culture containing a like amount of alcohol without drug was carried. The inoculum consisted of 0.05 ml of a 36- to 40-hour culture containing 100,000 trichomonads. The cultures were incubated at 37°C and

* The technical assistance of Mrs. Mary Williams, Miss Ruth Grossman, Mr. Pasquale Russo, and Mr. LeRoy Markle is hereby acknowledged. We wish to thank the staff of the Division of Bacteriology and Serology of the State of New Jersey Department of Health, Trenton, for generous amounts of human blood serum.

¹ Kupferberg, A. B., Johnson, G., and Sprince, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 304.

² Johnson, G., Trussell, M., and John F., *Science*, 1945, **102**, 126.

[†] The three strains of *T. foetus* used in this study were supplied by Dr. B. B. Morgan, University of Wisconsin.

Volatile Preservatives for Culture Media.

S. H. HUTNER AND CLARA A. BJERKNES. (Introduced by Paul A. Zahl.)

From the Haskins Laboratories, New York City.

During investigations of microbial nutrition centering about growth factors and trace elements, contaminants were repeatedly detected growing in stock solutions of many cp inorganic and organic compounds even when the solutions had been treated with toluene and chloroform, and stored at 6°C. The need for more effective preservatives motivated the study herein reported.

The use of volatile antiseptics is of long standing, especially in work with enzymes. Yet few comparative studies in this field have been described. The common alkyl halide solvents were the compounds tested in some typical studies.^{1,2} Propylene oxide and ethylene oxide were recently proposed for sterilizing culture media not to be subjected to heating.³

As the solutions to be protected were for use in compounding culture media sterilized by steam, completeness of volatilization of preservative was essential. For the past year, the addition of a mixture of *o*-fluorotoluene, *n*-butyl chloride and 1,2-dichloroethane, has enabled us to store putrescible solutions more or less indefinitely at refrigerator temperatures, and also at room temperature. A description of the development of this preservative may be helpful to others confronted with a similar problem, and may indicate some interesting possibilities in the field of volatile antiseptics.

Materials and Methods. a. *Compounds.* The following considerations guided selection of compounds to be tested:

1. Boiling point < 121°C.
2. Immiscibility with water to ensure removal by steam distillation on autoclaving.
3. Satisfactory chemical stability in con-

tact with water at room and sterilization temperature.

4. Low reactivity towards most compounds used in culture media. Where some decomposition might occur, the products to be nutritionally inert.

5. Moderate cost and ready availability.

At the beginning of this investigation a specific gravity near 1.0, to allow a more stable dispersion in dilute aqueous media, was considered a desideratum, but later experiments indicated advantages in blends of light and heavy compounds, and hence less importance was attached to this property. Compounds boiling at or below room temperature were too easily lost from opened containers. Hydrocarbons were excluded because they were too easily metabolized by microorganisms.⁴ Nevertheless because the use of toluene was traditional in enzyme studies, some preliminary experiments with it were conducted. It proved very inefficient: toluene-saturated broth allowed growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Torula utilis*; however it did check more or less completely *Bacillus cereus* and *Mycobacterium lacticola*. It was decided to concentrate attention on alkyl and aryl halides because these appeared best to fulfill the above listed requirements and they already had shown some effectiveness. Few tests were conducted with compounds with allylic halogen atoms because of their instability. Some sulfur-containing compounds were investigated despite their instability and unpleasant odors. Nearly all compounds tested were redistilled in an all-glass apparatus. "Practical" grade chemicals were distilled over a 3° range; nearly all other chemicals boiled over a much narrower range, agreeing well with the constants in the litera-

¹ Joachimoglu, G., *Biochem. Z.*, 1921, **124**, 130.

² Lihnell, D., *Arch. Mikrobiol.*, 1935, **6**, 326.

³ Hansen, H. N., and Snyder, W. C., *Phytopath.*, 1947, **37**, 369.

⁴ Zobel, C. E., *Bact. Revs.*, 1946, **10**, 1.

luxuriantly in the same Simplified Trypticase-Serum medium.

In an effort to determine whether the inhibitory effect of SN 13592 against *T. vaginalis* could be reversed with added Ca-pantothenate, 10 ml of media containing 1:200,000 drug with and without 320 µg of Ca-pantothenate were inoculated. No surviving cells were found in the absence of the Ca-pantothenate. With the added pantothenate the experimental population was equal to that in the control. It is thus suggested that, as with other microorganisms, the analogue of pantothenic acid acts in competition with the natural compound.

Due to its high level of *in vitro* activity it was thought worthwhile to submit SN 13592 to a study of its possible therapeutic activity in infected monkeys. Studies on the pharmacology of the compound by Singher, Millman, and Bosworth⁴ made possible a study of the therapeutic effect in cases of human vaginal trichomoniasis.[†]

Three monkeys, each weighing 5.5 kg, were successfully infected with a recently isolated strain (No. 9) of *T. vaginalis* which was obtained from a patient with an acute vaginitis.

Monkey No. 1 received 8 doses of 5.5 mg intravenously, followed by 24 oral doses of 300 mg administered twice daily. She remained infected and was therefore treated vaginally. Seven 300 mg doses were administered per vagina. Subsequent microscopic examination of the vaginal fluids revealed a persisting infection.

Monkey No. 2 received 9 oral doses of 275 mg given once daily. The infection persisted in spite of treatment. Seven vaginal doses of 300 mg were then administered without elimination of the infection.

Monkey No. 3 was given a long series of

graded oral doses, given twice daily as follows: 10 doses each of 10 mg, 20 mg, 40 mg, and 80 mg, followed by 9 doses of 160 mg, and 2 320-mg doses. The total drug administered amounted to 3.6 g. As in the other 2 monkeys, the infection was not eliminated.

In a series of 6 clinical cases of *Trichomonas vaginalis* vaginitis, a course of vaginal tablets containing 1 mg of SN 13592 was administered twice daily for 2 weeks. All 6 patients retained their infections in the face of the estimated 1:3000 dilution of the drug.

The failure of this analogue of pantothenic acid to eradicate vaginal trichomonad infections is referable to the pantothenic acid content of the blood serum. Denko, Grundy, and Porter⁵ demonstrated an average blood concentration of 33 µg per 100 ml. McIlwain and Hawking⁶ reported a sensitivity of staphylococci and streptococci to pantooyltaurine. Mice infected with these bacteria were not protected by pantooyltaurine. However, rats which have a lower blood level of pantothenic acid survived such infections when given a protective dose of pantooyltaurine. Woolley and White⁷ were unable to produce a pantothenate deficiency in mice by long continued administration of the same compound.

Conclusions. 1. The *in vitro* effectivity of several analogues of pantothenic acid has been demonstrated against *T. vaginalis*. 2. Wide variations in susceptibility of different strains to SN 13592 were found. 3. *T. foetus* and *T. gallinae* were found highly sensitive to this compound. 4. The *in vitro* activity of SN 13592 was reversed by the addition of calcium pantothenate. 5. *In vivo* studies in monkeys and in human beings demonstrated failure of SN 13592 to eradicate the infection.

⁵ Denko, W. D., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

⁶ McIlwain, H., and Hawking, F., *Lancet*, 1943, **1**, 459.

⁷ Woolley, D. W., and White, A. G. C., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 106.

⁴ Singher, H. O., Millman, N., and Bosworth, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 388.

[†] The authors are indebted to C. E. Folsome, M.D., for these clinical trials.

Volatile Preservatives for Culture Media.

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During investigations of microbial nutrition centering about growth factors and trace elements, contaminants were repeatedly detected growing in stock solutions of many cp inorganic and organic compounds even when the solutions had been treated with toluene and chloroform, and stored at 6°C. The need for more effective preservatives motivated the study herein reported.

The use of volatile antiseptics is of long standing, especially in work with enzymes. Yet few comparative studies in this field have been described. The common alkyl halide solvents were the compounds tested in some typical studies.^{1,2} Propylene oxide and ethylene oxide were recently proposed for sterilizing culture media not to be subjected to heating.³

As the solutions to be protected were for use in compounding culture media sterilized by steam, completeness of volatilization of preservative was essential. For the past year, the addition of a mixture of *o*-fluorotoluene, *n*-butyl chloride and 1,2-dichloroethane, has enabled us to store putrescible solutions more or less indefinitely at refrigerator temperatures, and also at room temperature. A description of the development of this preservative may be helpful to others confronted with a similar problem, and may indicate some interesting possibilities in the field of volatile antiseptics.

Materials and Methods. a. *Compounds.* The following considerations guided selection of compounds to be tested:

1. Boiling point < 121°C.
2. Immiscibility with water to ensure removal by steam distillation on autoclaving.
3. Satisfactory chemical stability in con-

tact with water at room and sterilization temperature.

4. Low reactivity towards most compounds used in culture media. Where some decomposition might occur, the products to be nutritionally inert.

5. Moderate cost and ready availability.

At the beginning of this investigation a specific gravity near 1.0, to allow a more stable dispersion in dilute aqueous media, was considered a desideratum, but later experiments indicated advantages in blends of light and heavy compounds, and hence less importance was attached to this property. Compounds boiling at or below room temperature were too easily lost from opened containers. Hydrocarbons were excluded because they were too easily metabolized by microorganisms.⁴ Nevertheless because the use of toluene was traditional in enzyme studies, some preliminary experiments with it were conducted. It proved very inefficient: toluene-saturated broth allowed growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Torula utilis*; however it did check more or less completely *Bacillus cereus* and *Mycobacterium lacticola*. It was decided to concentrate attention on alkyl and aryl halides because these appeared best to fulfill the above listed requirements and they already had shown some effectiveness. Few tests were conducted with compounds with allylic halogen atoms because of their instability. Some sulfur-containing compounds were investigated despite their instability and unpleasant odors. Nearly all compounds tested were redistilled in an all-glass apparatus. "Practical" grade chemicals were distilled over a 3° range; nearly all other chemicals boiled over a much narrower range, agreeing well with the constants in the litera-

¹ Joachimoglu, G., *Biochem. Z.*, 1921, **124**, 130.

² Lilhnell, D., *Arch. Mikrobiol.*, 1935, **6**, 326.

³ Hansen, H. N., and Snyder, W. C., *Phytopath.*, 1947, **37**, 369.

⁴ Zobell, C. E., *Bact. Revs.*, 1946, **10**, 1.

TABLE I.
Compounds Tested.

Toluene	1-bromo-2-methyl-propane (<i>iso</i> -butyl bromide)
Diethyl ether	4-bromo-2-methyl butane (<i>iso</i> -amyl bromide)
Iso-propyl ether	1,2-dibromoethylene
Carbon tetrachloride	1,1-dibromoethane (ethylidene bromide)
Chloroform	1-bromo-1-propene
Dichloromethane (methylene chloride)	2-bromo-1-chloro-propane
1-chloropropane (<i>n</i> -propyl chloride)	chlorobromomethane
2-chloropropane (<i>iso</i> -propyl chloride)	1,2-chlorobromoethane (1,2-ethylene chlorobromide)
1-chlorobutane (<i>n</i> -butyl chloride)	Iodoethane (ethyl iodide)
Monochlorobutenes	1-iodo-propane (<i>n</i> -propyl iodide)
2-chloro-2-methyl-1-propane (<i>t</i> -butyl-chloride)	2-iodo-butane (<i>sec</i> -butyl iodide)
4-chloro-2-methyl butane (<i>iso</i> -amyl chloride)	<i>p</i> -fluorotoluene
1-chlorohexane (<i>n</i> -hexyl chloride)	<i>o</i> -fluorotoluene
1,1-dichloroethane (ethylidene chloride)	Fluorobenzene
1,2-dichloroethane (ethylene chloride)	Benzotrifluoride
1,1,1-trichloroethane (methyl chloroform)	1,1,2-trichloro-3-trifluoro-1-propene
1,2-dichloroethylene	Thiophene
1,1,2,2-tetrachlorethylene (carbon dichloride)	2-methyl thiophene
1-chloro-1-propene	3-methyl thiophene
Bromoethane (ethyl bromide)	Dimethyl disulfide
1-bromo-1-butane (<i>n</i> -butyl bromide)	Methyl ethyl sulfide
2-bromo-2-methyl propane (<i>t</i> -butyl bromide)	Methyl orthoacetate

TABLE II.
Test Organisms

Routine test organisms	Source
<i>Escherichia coli</i>	R. R. Roepke ATCC* 9723
<i>Protaminobacter albobiflavum</i> b	C. B. van Niel
<i>Pseudomonas riboflavina</i>	J. W. Foster (available from ATCC)
<i>Corynebacterium pseudodiphthericum</i>	ATCC 6951
<i>Staphylococcus aureus</i>	" 9144
<i>Streptococcus faecalis</i>	" 8043
<i>Actinomyces scabies</i>	" 3021
<i>Bacillus subtilis</i>	" 6633
<i>Torula utilis</i>	" 8206

* ATCC—American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C.

ture and commercial catalogs. The compounds tested are listed in Table I. For the sake of brevity, source and physical constants are omitted. Most of the chemicals were obtained either from the Eastman Kodak Co., the Amend Drug and Chemical Co., or the Columbia Organic Chemical Co., Inc., Columbia, S.C. The methyl chloroform and thiophenes were gifts of the Dow Chemical Co. and the Socony-Vacuum Laboratories respectively. Where alternate names are in use, the one employed by *Chemical Abstracts* is given first.

b. *Test Organisms*. The organisms were selected to represent some of the genera commonly encountered as air contaminants in the ordinary course of laboratory work. Mycelial fungi were not included because they never appeared in preservative-treated solutions. It

was supposed that acid-fast bacteria might be important contaminants, but they were not encountered, and hence a petroleum-utilizing strain of *Mycobacterium lacticola* and a rapidly-growing strain of *M. avium* were dropped from the roster of routine test organisms. They were killed by nearly all the halogenated compounds tried. The organisms selected for routine use are listed in Table II. Additional organisms were used in later tests.

c. *Test Procedure*. Ten ml amounts of broth, whose composition is given in Table III, were distributed in 125 x 20 screw-capped tubes and sterilized for 20 minutes at 117 to 121°C. The medium allowed heavy rapid growth at room temperature of all the routine test organisms. The tubes were inoculated with a drop of fresh slant growth suspended in broth. After 4 hours at room temperature,

TABLE III.
Composition of Test Medium.

	%
K ₂ HPO ₄	0.05
MgSO ₄ · 7H ₂ O	0.01
Na ₂ citrate · 2H ₂ O	0.1
Trypticase*	0.6
Lactate	0.25
Glycerol	0.5
Solubilized Liver†	0.1
pH 6.9 to 7.1	

* Baltimore Biological Laboratory.

† Wilson's Liver "L."

the compounds to be tested were aseptically pipetted in, to a final concentration by volume of 1%. The tubes were shaken vigorously and then allowed to stand at room temperature (21 to 28°C) for 96 to 120 hours. Growth was then recorded. To test for completeness of removal of the preservative on autoclaving, and absence in the medium of toxic residual decomposition products, the materials under test were added to broth, which was then autoclaved according to the routine technic. With this method, after sterilization, the exhaust valve of the autoclave was kept shut until the partial vacuum which develops on cooling was subsequently dissipated by the gradual influx of air. If necessary the exhaust valve was opened very slightly. This procedure was equivalent to steam distillation under reduced pressure and no difficulty was experienced in complete removal of preservatives, even in amounts well over the 1% level. To illustrate the effectiveness of the method, trials with *n*-hexyl chloride (b.p. 132.6°C) showed it completely removed under the standard conditions. In orientation experiments it was convenient to test for non-volatilized preservative by adding a few drops of I-KI solution; free iodine dissolving in the preservative made tiny droplets conspicuous.

Mixtures were compounded on a volume basis. In practice, a few drops of preservative were added to the solution in a glass-stoppered bottle and the bottle was then shaken vigorously. Volumes of culture media up to 2 liters were thus successfully preserved for months in the refrigerator.

Results. After conducting killing tests in this manner on 16 compounds, the majority of them halogenated hydrocarbons, it ap-

TABLE IV.
Killing Tests with Mixtures of *o*-Fluorotoluene, *n*-Butyl Chloride, and 1,2-Dichloroethane.

	Routine No. 3*	<i>o</i> -Fluoro- toluene	<i>n</i> -Butyl chloride	1,2-dichloro- ethane	<i>o</i> -Fluorotoluene- <i>n</i> -Butyl chloride- 1,2-dichloroethane		
					1:1:1	1:2:1	1:3:1
<i>Escherichia coli</i>	+	0	+	+	0	0	0
<i>Protaminobacter alboblaum b</i>	+	0	+	+	0	0	0
<i>Pseudomonas riboflavina</i>	+	0	+	+	0	0	0
<i>Corynebacterium pseudodiphthericum</i>	+	0	+	+	0	0	0
<i>Staphylococcus aureus</i>	+	+	+	+	0	0	0
<i>Streptococcus faecalis</i>	+	+	+	+	0	0	0
<i>Actinomyces scabicus</i>	+	0	+	+	0	0	0
<i>Bacillus subtilis</i>	+	0	+	+	0	0	0
<i>Torula utilis</i>	+	+	+	+	0	0	0
* Routine No. 3 33% 1-Bromo-1-propene 67% <i>n</i> -Butyl chloride	0 = growth + = growth						

peared that a 3:1 mixture of *n*-butyl chloride and CCl_4 might be satisfactory. Need for a better preservative later became evident, since contaminations of stock solutions, while much less frequent, still appeared; and under test conditions there was some growth of *E. coli*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Torula utilis*. The next mixture extensively used consisted of equal parts of CCl_4 , *n*-butyl chloride, and 1,2-dichloroethane. This was much more effective but did not completely check the growth of *E. coli* and *Strep. faecalis*. Complete effectiveness was shown by a mixture of 1 part 1-bromo-1-propene and 2 parts *n*-butyl chloride. This not only completely inhibited the routine test organisms and many others, but no contaminants appeared in practice; 1-bromo-1-propene was not altogether stable, yellowing slowly at room temperature. This did not significantly alter its antiseptic properties, and volatility tests with yellowed samples remained satisfactory. However on continued use it became extremely irritating to the eyes and nose, and had to be discarded. Several of the somewhat less effective halides were tried singly, and many were tried in combination with *n*-butyl chloride and 1,2-dichloroethane. *O*- and *p*-fluorotoluene appeared unusually effective in repeated trials and were then tested in detail in combination with other halides. The *o*-isomer appeared somewhat more effective and is part of the preservative now in use. A typical experiment with the present preservative is shown in Table IV. About 50 experiments in all were performed on this scale.

The sulfur compounds were almost completely non-inhibitory. No consistent differences emerged between bromo and chloro homologues. The iodo derivatives were not effective enough and were too unstable to encourage further work in that direction.

The preservative finally adopted consisted of a mixture of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichloroethane.

Discussion. Rhodotorulae had previously been isolated as principal contaminants of solutions preserved with the less effective alkyl halide-containing mixtures. Their abundant growth in such seemingly unfavorable media

as 3% boric acid has been described.⁵ They have not appeared in any of the solutions preserved with the new mixture. Exposure of varied agar plate media indicated that rhodotorulae were quantitatively insignificant but constantly present in our laboratory air. As they have been reported to be pyrogenic,⁶ they may perhaps constitute an important source of pyrogenic contamination of therapeutic preparations.

Certain limitations attend the use of alkyl and aryl halide preservatives. Compounds of this class react slowly with $-\text{SH}$ groups.⁷ This does not appear to affect the practical usefulness of these compounds in preservation of natural materials such as yeast extract and protein hydrolysates, probably because most of the potential $-\text{SH}$ compounds are in the disulfide form. This reaction became evident when concentrated solutions of Na thiosulfate were treated with the preservative: the solutions became toxic and acquired peculiar odors. Slow interaction with $-\text{SH}$ compounds may be of significance in accounting for the effectiveness of the preservative. Little more can be said at present as to their mode of action. In the recommended preservative mixtures, the most effective component is *o*-fluorotoluene. The *n*-butyl chloride, lighter than water, is a poor preservative but serves to bring the specific gravity of the mixture closer to that of water, besides being an inexpensive extender for the relatively expensive *o*-fluorotoluene. The 1,2-dichloroethane may function as a contact agent because of its relatively good solubility in water (ca. 0.9% at room temperature) bringing the less soluble *o*-fluorotoluene into more effective contact with the cell. The obvious fat-solvent properties of these compounds appear inadequate to account for their striking differences in effectiveness.

The tremendous recent expansion in the

⁵ Skinner, C. E., et al., 2nd ed. *Henrici's Molds, Yeasts, and Actinomyces*, 1947, John Wiley and Sons, N.Y.

⁶ Co Tui, F. W., and Schrifft, M. H., *J. Lab. Clin. Med.*, 1942, **27**, 569.

⁷ Hickinbottom, W. J., *Reactions of Organic Compounds*, p. 106, Longmans, Green and Co., 1936, New York.

laboratory and industrial chemistry of organic fluorine compounds⁸ may, in the light of the results reported here, encourage further work in the rather neglected field of the water-immiscible volatile antiseptics.

⁸ Symposium on Organo-fluorine Chemistry, 1947, *Ind. Eng. Chem.* (many authors), **39**, 235.

Summary. A mixture by volume of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichlorethane, is recommended as a chemically nearly inert but nevertheless effective preservative for biological solutions. This preservative, used at approximately the 1% level, is completely removed under the usual conditions of steam sterilization.

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Isolation of an Agent in Chicken Embryo Causing Infectious Sinusitis of Turkeys.

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The term infectious sinusitis of turkeys was suggested by Dickenson and Hinshaw¹ to differentiate it from sinusitis which may occur in connection with reduced vitamin A intake² or follow inoculation with *Hemophilus gallinarum*.³ Infectious sinusitis occurs in all sections of the country and although mortality is low, morbidity varies from 10% to 90% of a flock.^{1,4} Thus, losses to the grower resulting from inferior quality of the birds are often quite heavy. According to the literature Delaplane⁵ has reported a *Pasteurella*-like organism to be associated with an infectious type of sinusitis. In transmission experiments Hart⁶ and Hinshaw⁷ were unable to filter the causal agent through collodion membranes, Chamberland or sintered glass filters.

Serial transmission of the disease in this

laboratory from a case of sinusitis submitted to the laboratory for examination was carried out in the usual manner.⁷ Sinus exudate was aspirated from infected turkeys and 0.5 ml amounts were inoculated into the right infra-orbital sinus of normal turkeys. The inoculated sinus became swollen 5 to 10 days after inoculation and contained a viscous exudate which resembled egg albumen and was bacteriologically sterile. Later the sinuses became greatly distended and often contained caseated material from which a variety of bacteria were isolated. On the assumption that these organisms represented secondary bacterial invaders an attempt was made to isolate the causal agent early in the course of the disease from the third serial turkey passage. Accordingly, sinus exudate was aspirated on the second day after the appearance of clinical symptoms and was inoculated into embryonating eggs by the following routes: onto the chorioallantois, into the allantoic cavity and into the yolk sac. No embryo deaths or significant macroscopic lesions were observed in any of the eggs inoculated onto the chorioallantois or into the allantoic cavity. However, all of the 10 embryos inoculated into the yolk sac died 6 to 12 days after inoculation. Yolk sacs from these embryos were bacteriologically sterile and were subsequently passed in

¹ Dickenson, E. M., and Hinshaw, W. R., *J. Am. Vet. Med. Assn.*, 1938, **93**, 151.

² Hinshaw, W. R., and Lloyd, W. E., *Hilgardia*, 1934, **8**, 281.

³ Beach, J. R., and Schalm, O. W., *Poult. Sc.*, 1936, **15**, 466.

⁴ Lee, C. D., No. *Am. Vet.*, 1942, **23**, 715.

⁵ Delaplane, J. P., *Poult. Sc.*, 1944, **23**, 247.

⁶ Hart, L., *Austral. Vet. J.*, 1940, **16**, 163.

⁷ Hinshaw, W. R., from *Diseases of Poultry*, edited by H. E. Biester and L. Devries, The Iowa State College Press, 1945.

chicken embryos by yolk sac inoculation for a total of 28 consecutive passages.

After the second yolk sac passage 0.5 ml of a 10% yolk sac suspension was inoculated into the right sinus of each of 2 normal turkeys. The sinuses became swollen 5 and 8 days after inoculation respectively. Sinus exudate aspirated on the first day after the appearance of clinical symptoms was bacteriologically sterile and killed all of 10 chicken embryos 6 to 12 days after inoculation into the yolk sac. Clinical sinusitis was produced in a total of 10 additional turkeys with yolk sac material from the 3rd, 4th, and 16th egg passages respectively.

Filtration studies on the egg-adapted strain of the agent of turkey sinusitis were carried out using a 1/100 dilution of infected yolk sacs. At least 50.0 ml amounts of the suspension were filtered through Berkefeld V, Berkefeld N, and Seitz E K filters respectively. The filtrates obtained, together with the unfiltered suspension, were tested for infectivity by inoculation into groups of 15 embryonating eggs. All embryos inoculated with the unfiltered suspension and the Berkefeld V filtrate died 4 to 8 days after inoculation. However, all embryos inoculated with Berkefeld N and Seitz E K filtrates respectively were still living 14 days after inoculation. It is evident that the causal agent passed through a Berkefeld V filter but did not pass readily Berkefeld N or Seitz E K filters.

During the first 10 egg passages micro-

scopic examination of impression smears of infected yolk sacs stained by Machiavello's¹ technic revealed only an occasional red staining coccoid body. However, beginning with the 14th egg passage when the infective titer of yolk sacs reached 10^{-8} or more small, coccoid bodies were regularly observed both intra- and extracellularly. These bodies were found to vary in size from approximately 0.5μ to 1.0μ or more in diameter and were frequently imbedded in a matrix. The bodies described above closely resembled morphologically the bodies found in similar impression smears of yolk sacs infected with the etiological agents of the lymphogranuloma-psittacosis group (*Chlamydozoaccae*).

Further studies on the characteristics of the agent of turkey sinusitis and its possible etiological relationship to an agent isolated from air sac infection in turkeys by Minard and Jungherr⁸ are in progress.

Summary. 1. An agent capable of producing clinical sinusitis in turkeys was isolated and propagated in the yolk sac of the developing chicken embryo. 2. Impression smears of infected yolk sacs stained by Machiavello's technic revealed morphologic forms closely resembling those found in the lymphogranuloma-psittacosis group of agents (*Chlamydozoaccae*).

⁸ Minard, E. L., and Jungherr, E., 1944, Proc. 16th An. Conf. Lab. Workers in Pullorum Dis. Control, Coll. of Agr., Univ. of Conn., mimeog. report.

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Observations on the Effect of 4-Amino-Pteroylglutamic Acid on Mice.

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The production of a syndrome in mice by feeding a crude "antagonist" of pteroylglutamic acid (PGA) and its prevention by raising the dietary level of PGA was described.¹ The syndrome was marked by slow growth followed by a loss in weight, low hemoglobin

and low white blood cell count. Recently, another substance, "4-amino" pteroylglutamic acid ("4-amino-PGA"), which is strongly

¹ Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1947, 65, 368.

antagonistic to PGA in the *Streptococcus fecalis* R test, was described.² This compound differs from PGA only in having an amino group in place of the hydroxyl group on the pteridine ring. Its inhibition of the growth of *S. fecalis* R was reversed by increasing the level of PGA. The effect of the compound in the nutrition of mice was studied in the present investigation.

Experimental. The animals received a purified diet without PGA and with 1% succinylsulfathiazole, identical with the diet previously described¹ except that *p*-aminobenzoic acid was omitted in Experiment 2. In a preliminary experiment it was found that mice died within a few days after being placed on a diet containing levels of 1 mg of 4-amino-PGA or more per kilo. Accordingly in Experiment 1 low levels of 4-amino-PGA were fed with various amounts of PGA ranging up to comparatively high levels. The results are in Table I. At a level of 0.3 mg the 4-amino compound had a slight tendency to depress the blood values which was reversed by high levels of PGA. When the level of the compound was raised to 1.0 mg, mortality was high even with large amounts of PGA.

In Experiment 2, the effects of PGA upon mice receiving 4-amino-PGA was explored further. Higher levels of 4-amino-PGA were used, ranging up to 3 mg per kilo of diet. Some reversal of the toxic effect was observed at the 0.3 mg level, but no protection was afforded by PGA when the 4-amino compound was fed at levels of 1 or 3 mg per kilo of diet (Table II).

No lesions were visible upon gross examination at autopsy.

Summary. The 4-amino analogue of pteroylglutamic acid was fed to mice as a dietary supplement. There was some indication of reversal by pteroylglutamic acid of the effect on mice when the level of the antagonist was 0.3 part per million of diet. Death was found to occur within a few days with levels of 1 part per million of diet or higher. The effect was not reversed by feeding high levels

TABLE I.
Effects of Various Levels of PGA and 4-Amino PGA on Growth and Hematology of Mice. Six Mice Were Used in Each Group.

Group	Supplement in mg per kilo of diet		Body wt and No. of survivors					Hemoglobin g per 100 cc		White cells ($\times 10^3$) per mm ³	
	PGA	4-amino PGA	0	1	2	4	5 wk	at 2 wk	at 4 wk	at 2 wk	at 4 wk
1	0.1	0	11	16	17	21 (6)	21 (5)	10.3	13.0	7.1	10.0
2	1.0	0	11	16	17	21	22 (6)	14.3	14.4	12.5	9.4
3	10	0	11	16	18	21	22 (6)	16.2	17.6	8.8	11.8
4	100	0	12	18	19	23	23 (6)	13.7	16.4	9.0	13.0
5	0.1	0.1	13	17 (5)	18	20	19 (5)	11.3	12.2	11.4	7.3
6	0.1	0.1	12	18	21	26	26 (6)	15.8	15.8	11.8	13.4
7	1.0	0.1	12	18	21	25 (5)	24 (4)	13.4	15.8	13.2	11.8
8	100	0.1	12	17	23	23	24 (6)	15.6	16.9	12.8	10.9
9	0.1	0.3	13	16	15 (5)	20	20 (5)	8.5	10.4	7.4	5.6
10	1.0	0.3	12	14 (5)	16	20	21 (5)	13.9	13.4	15.3	9.1
11	10	0.3	12	19	22	24	24 (6)	14.1	15.7	14.4	11.9
12	100	0.3	13	18	20	22	21 (6)	15.7	17.2	14.5	16.8
13	10	1.0	13	12 (3)	14 (1)	22 (0)	23 (0)	12.1	—	10.3	—
14	100	1.0	11	14 (2)	17 (2)	22 (2)	23 (2)	10.9	11.5	7.0	10.2

² Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, 69, 2567.

TABLE II.
Effects of PGA and 4-Amino PGA on Growth and Survival of Mice in Experiment 2. Five mice were used per group.

Group	Supplement in mg per kg of diet		Body wt and (in parentheses) No. of survivors					Avg survival time in days
	PGA	4-amino PGA	0	1	3	5	8 wk	
15	0.1	0	10	14	21	24	22 (5)	
16	1.0	0	10	13	19	19	19 (5)	
17	10	0	10	15	20	22	24 (5)	
18	100	0	11	13	20	22	24 (5)	
19	0.1	0.1	10	15	20	24	24 (5)	
20	1.0	0.1	10	16	21	26	28 (5)	
21	10	0.1	10	16	21	23	24 (5)	
22	100	0.1	11	16	23	26	30 (5)	
23	0.1	0.3	10	(0)				4
24	1.0	0.3	10	13 (4)	20	23	26 (4)	
25	10	0.3	10	13	18	20	21 (5)	
26	100	0.3	9	14	18 (4)	21	24 (4)	
27	0.1	1.0	10	9 (1)	(0)			5
28	1.0	1.0	10	13 (1)	(0)			8
29	10	1.0	10	9 (1)	(0)			7
30	100	1.0	10	(0)				5
31	0.1	3.0	10	(0)				6
32	1.0	3.0	11	(0)				4
33	10	3.0	10	(0)				4
34	100	3.0	10	(0)				4

* In the case of groups in which all the animals died during the experimental period.

of pteroylglutamic acid. This finding may be contrasted with the inhibitory effect of the 4-amino compound on *S. fecalis* R which reversed by pteroylglutamic acid.

16321

Relation Between Induced Hyperthyroidism and an Unidentified Chick Growth Factor.*

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The use of biological assays for study of unidentified growth factors is often restricted by the extent to which they can measure low concentration of a factor in a given material. This may be due to the small response of

the animal to a given supplement. A series of studies have shown that the chick require an unidentified growth factor found in condensed fish solubles,¹ whole liver powder, and certain other liver fractions.^{2,3} The increased growth rate of the chick as a result of adding supplements carrying the factor has been used as an assay in determining some of the prop-

* Published with the approval of the Director of the Wisconsin Experiment Station. Supported in part by grants from the Borden Company, New York City, and the Commercial Solvents Corporation, Terre Haute, Ind.

¹ Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Science*, 1948, in press.

² Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *Poultry Science*, 1948, in press.

³ Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

TABLE I.
 Effect of Supplements on the Growth Rate of Chicks.

Exp. No.	Lot No.	Supplements to basal ration	Avg wt at 4 wks	Mortality %
1	1	None	120	25
	2	3% condensed fish solubles	220	0
	3	.25% desiccated thyroid U.S.P.	129	42
	4	.25% " " + 3% condensed fish solubles	203	25
	5	.25% " " + .05 cc Reticulogen (Lilly)/bird/day*	171	25
2	6	None	178	0
	7	3% condensed fish solubles	249	0
	8	.125% desiccated thyroid	137	0
	9	.125% " " + 3% condensed fish solubles	279	0
3	10	None	141	17
	11	3% condensed fish solubles	205	0
	12	.03% iodinated casein	96	33
	13	.03% " " + 3% condensed fish solubles	238	8
4	14	None	129	15
	15	3% condensed fish solubles	191	15
	16	.125% desiccated thyroid	134	40
	17	.125% " " + 3% condensed fish solubles	221	0
	18	.02% iodinated casein	117	40
	19	.02% " " + 3% condensed fish solubles	232	5
5	20	None	159	0
	21	3% condensed fish solubles	259	0
	22	.05 cc Reticulogen/bird/day*	240	0
	23	.125% desiccated thyroid	156	17
	24	.125% " " + 3% condensed fish solubles	249	17
	25	.125% " " + .05 cc Reticulogen/bird/day*	233	0
	26	.02% iodinated casein	122	8
	27	.02% " " + 3% condensed fish solubles	273	0
	28	.02% " " + .05 cc Reticulogen/bird/day*	251	0

* Injected.

erties of the factor.⁴ However, in more recent work (unpublished) with low concentrations of the factor the response obtained is sometimes such that it is questionable whether it may be attributed to the presence of the factor, or merely to normal variation between the experimental groups of chicks.

The recent work of Bethell, Wiebelhaus, and Lardy⁵ and of Ershoff *et al.*⁶ offers a method which may be helpful in improving certain biological assays for unidentified growth factors. They found that the admin-

istration of desiccated thyroid to rats resulted in poor growth and a shortened survival period. It was suggested that feeding desiccated thyroid increases the requirement of the growing animal for some unknown factor(s), and that this increased requirement is fulfilled by feeding materials containing the unidentified antithyrototoxic factor. Liver and yeast were found to be sources of the antithyrototoxic material. It has been long recognized that hyperthyroidism increases the animals' requirement for various nutrients. The relationship of experimentally induced hyperthyroidism to increased requirements of many of the known vitamins has been reviewed.⁵

The observations of Bethell *et al.*⁵ suggested that production of a condition of hyperthyroidism in chicks might be useful in

⁴ Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Biol. Chem.*, 1948, 173, 117.

⁵ Bethell, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, 34, 431.

⁶ Ershoff, B. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 500.

increasing the effective assay range, and sensitivity of chicks to the unidentified chick growth factor found in condensed fish solubles and certain liver fractions. The present investigations were initiated to study the effect of thyroid active materials on the degree of response of chicks to supplements containing the unidentified factor or factors.

Experimental. Day-old (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) chicks were used in all the studies. The chicks were progeny of hens fed diet B1, described in a previous report.¹ The experimental groups were housed in electrically heated batteries with raised screen floors. The chicks were wing-banded and weighed at the beginning of the experiment and at weekly intervals thereafter. Feed and water were supplied *ad libitum*. The experiments were terminated at the end of four weeks. The basal diet used was the same as that used in previous trials.¹ It consisted of the following ingredients: ground yellow corn 35, wheat bran 10, wheat middlings 10, dehydrated alfalfa meal 5, soybean oil meal 28, vitamin test casein 7.5, limestone grit 2.0, steamed bonemeal 1.5, iodized salt 0.5, fish oil (2000A-400D) 0.5, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.025 g; thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxine HCl 0.4, inositol 100, choline chloride 150, *p*-aminobenzoic acid 10, biotin 0.02, folic acid 0.05, menadione 0.05, α -tocopherol 0.3 mg. Supplements to the basal ration were made at the expense of the corn.

Results. The results obtained are presented in Table I. In trial 1 the addition of 0.25% desiccated thyroid to the basal diet resulted in slow growth and high mortality (lot 3). The growth rate in this case was not significantly different from that of the basal group (lot 1). The addition of either 3% condensed fish solubles to the diet containing desiccated thyroid; or the daily injection of 1 U.S.P. unit of reticulogen (Lilly), a concentrated liver extract used in the treatment of pernicious anemia, gave a significant increase in growth rate. In both cases, however, the average weight attained at 4 weeks of age was somewhat below that of the positive control group in which condensed fish solubles alone was added to the basal ration (lot 2).

In the second trial a lower level of desiccated thyroid was used. In this case, a response of .125% desiccated thyroid in the diet (lot 8) resulted in a significantly lower growth rate than was obtained with the basal group (lot 6). The addition of 3% condensed fish solubles and .125% desiccated thyroid to the basal diet (lot 9) resulted in a significant increase in growth above that of the positive control (lot 7) which was supplemented with fish solubles alone.

Another source of thyroïdal activity was tested in trial 3. Iodinated casein (3.07% thyroxine) was added to the basal ration at a level of .03%. A decrease in growth rate and an increase in mortality resulted (lot 12). The effect obtained when 0.03% iodinated casein and 3% condensed fish solubles were added was similar to that observed in the previous trial using desiccated thyroid. The average weight at 4 weeks of age (lot 13) was significantly above that of the positive control (lot 11).

The results in trial 4 are essentially in agreement with those of the previous 2 trials. The addition of desiccated thyroid to the basal ration (lot 16) did not result in a lower rate of growth than was obtained with the basal ration alone, but was accompanied with a marked increase in mortality. Iodinated casein (lot 18) caused both a decreased growth rate and high mortality. When the basal ration was supplemented with either of these materials along with 3% condensed fish solubles (lots 17, 19) a growth response greater than that which was obtained with condensed fish solubles alone (lot 15) resulted.

Trial 5 was carried out to determine whether or not reticulogen injections would fully substitute for condensed fish solubles in rations containing iodinated casein or .125% desiccated thyroid. The results show that reticulogen was effective in both cases (lots 25, 28) in overcoming the thyrotoxic effect of these materials. The administration of desiccated thyroid did not cause a decreased growth rate (lot 23). The growth rate was depressed in the group fed 0.2% iodinated casein (lot 26). In both cases mortality was higher than in the control groups. The addition of con-

fish solubles and iodinated casein gave a growth response, but this effect was observed with desiccated thyroid and fish solubles.

Discussion. The data presented here show that either condensed fish solubles or reticulogen (Lilly) serve to counteract the thyrotoxic effect of desiccated thyroid in the chick. This result parallels the observations of Betheil *et al.*⁵ and of Bethell *et al.*⁷ who noted an anti-thyrotoxic effect of liver and yeast when desiccated thyroid was fed to rats.

A similar protective action was also observed when iodinated casein was used in place of desiccated thyroid. This would indicate that the thyrotoxic effect is attributable to an increased thyroid activity. Since the protective action is exerted by both condensed fish solubles and reticulogen (Lilly) it would appear that the anti-thyrotoxic factor is identical to the unidentified growth factor described previously.^{1,2}

In addition to the protective action of condensed fish solubles a further effect was noted in these experiments. An increased growth rate was obtained when either 0.125% desiccated thyroid or .02-.03% iodinated casein was added to the ration containing condensed fish solubles. The effect is similar to that observed by Irwin, Reinecke and Turner⁷ who reported a stimulation in growth of chicks when iodinated casein was added to the ration. However, in the experiments reported herein the addition of either desiccated thyroid or iodinated casein to the basal ration without fish solubles caused a decreased growth rate and high mortality and it was only in the presence of an adequate amount of the factor supplied by condensed fish solubles that a growth stimulation was obtained.

The levels of iodinated casein that were found to be effective were considerably lower than those employed by Irwin *et al.*⁷ They reported that 36 g of iodo-casein (3.1% thyroxine) per cwt. of feed was the optimum dosage level and that variations from this level were not effective. In the experiments reported herein the effective levels ranged

from 9.08 to 13.62 g iodinated casein (3.07% thyroxine) per 100 lb. of feed. Higher levels were not tested. In a recent report Wheeler, Hoffman, and Graham⁸ found that a diet containing 10 g of thyroprotein (3.0% thyroxine) per cwt. of feed resulted in a significant increase in uniformity and mean body weight of male birds at 12 weeks of age. The level of iodinated casein used was within the range we found to be effective.

The growth stimulation obtained may be due to an increase in the basal metabolic rate of the chick. Evidence that the administration of thyro-active materials to animals causes an increase in the requirement of many nutrients has been adequately reviewed by Bethell *et al.*⁵ It appears that in the presence of sufficient amounts of all the nutrients, the increased basal metabolic rate results in a more rapid growth rate than is exhibited by the normal or untreated individual rather than a thyro-toxicity. It is quite evident, however, that a deficiency in the ration of the unidentified factor(s) found in condensed fish solubles results in a thyro-toxicity when desiccated thyroid or iodinated casein is administered.

The results that have been obtained by various investigators would seem to be dependent on 2 factors: the completeness of the ration and the level of thyro-active material fed. The ration used by Wheeler *et al.*⁸ containing both fish meal and meat meal as protein supplements, would likely meet both of these requirements; however the basal ration used by Turner, Irwin, and Reinecke⁹ and by Irwin *et al.*⁷ might be submarginal with regard to the unidentified factor of condensed fish solubles.

The results of the study indicate that an experimentally induced hyperthyroidism may be an extremely useful tool in biological assay for unidentified factor(s) required by the chick for optimum growth. A level in the diet of .125% desiccated thyroid or levels of .02 to .03% iodinated casein resulted in an

⁸ Wheeler, R. S., Hoffman, E., and Graham, C. L., *Poultry Science*, 1945, **27**, 103.

⁷ Irwin, M. R., Reinecke, E. P., and Turner, C. W., *Poultry Science*, 1943, **22**, 374.

⁹ Turner, C. A., Irwin, M. R., and Reinecke, E. P., *Poultry Science*, 1944, **23**, 242.

increase in the assay range for the unidentified factor.

The increase in growth rate which was obtained when iodinated casein or desiccated thyroid was added to the ration containing condensed fish solubles has not been studied beyond 4 weeks of age. Work is now in progress to determine the possible value of a combination of fish solubles and iodinated casein in commercial feeding.

Summary. A thyrotoxic condition in the chick, induced by feeding desiccated thyroid or iodinated casein was effectively counteracted either by supplementing the diet with condensed fish solubles or by the injection of reticulogen.

An increased growth response was obtained upon the addition of either desiccated thyroid or iodinated casein to a ration containing adequate amounts of the known and unidentified chick growth factors.

The addition of either 0.125% desiccated thyroid or 0.02 to .03% iodinated casein to the basal ration resulted in an improved assay range for the unidentified chick growth factor(s) in condensed fish solubles and reticulogen.

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16322

Some Characteristics of the Anti-Estrous Factor in *Lithospermum*.*

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Two laboratories have independently reported that the herb *Lithospermum ruderales* when mixed with normal diet abolishes the estrous cycle in mice.^{1,2} The present note describes further studies on modes of administration, site of action within the body, differential strain susceptibilities, distribution of the factor within the *Lithospermum* plant, as well as some preliminary fractionation work.

Experimental. 1. *Absorption from Pellets.* Daily vaginal smears, using the method previously described,² were made from large

groups of Rockland Swiss strain virgin female mice between 2 and 4 months of age. Fifty of these mice showing least irregularity of estrus were selected. These were further segregated into 5 uniform weight groups. One lot was maintained as the control, each mouse receiving a subcutaneously implanted 50 mg beeswax pellet. Individuals of the second lot received similarly implanted pellets consisting of 25 mg of beeswax and 25 mg of the crude oil residue of the ether extraction of 100 g of powdered *Lithospermum*.[†] A neutral water extract of the same batch of *Lithospermum* was incorporated in similar proportions into other beeswax pellets, and implanted into a third group of mice. A fourth group received beeswax pellets con-

* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service. Technical assistants on the project were M. L. Drasher and Andrew Nowak.

¹ Cranston, E. M., *J. Pharm. and Exp. Therap.*, 1945, **83**, 130.

² Drasher, M. L., and Zahl, Paul A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 66.

[†] *Lithospermum* powder was prepared by milling the whole plant, including roots, stems, floral parts, stalks, and leaves. Florescent plants were collected in mid-June in the deserts of western Montana, and dried in the shade.

taining 2.5 mg of *iso*-androliclactone acetate.[†] A fifth group received beeswax pellets containing 10 mg of thiouracil.[§] A daily estrous record for each of the mice comprising these groups had been kept for 20 days previous to pellet implantation; and vaginal smear estrous assay was continued for 40 days following the implantation. In only one of the 5 groups was there observable suppression of estrus, *i.e.*, the group receiving the water-soluble *Lithospermum* fraction.

The mean weights of each group of mice were taken weekly, starting with the time of pellet implantation and continuing during the experimental 40-day period. The weight curves were normal except for that of the thiouracil group, and to some extent that of the *iso*-androliclactone acetate group, which dropped somewhat initially, but recovered within 3 weeks. The drop in the thiouracil weight curve is taken to indicate that an effective anti-thyroid level of the compound was being maintained by absorption of the pellet, without exertion of any pronounced anti-estrous effect. It is not clear how thiouracil under these conditions would effect a weight loss; one may assume either some kind of side action, or assume that absorption from the pellet was so rapid as to constitute a gross overdose. Whether the *iso*-androliclactone acetate was similarly toxic was not clear, due to the questionable significance of its weight curve aberrancy.

2. *Strain Differences.* It was desirable to obtain additional data on strain differences in respect to susceptibility to the action of *Lithospermum*. The strains to be compared were the Rockland Swiss and the C₃H lines, the latter a high mammary tumor incidence strain. A set of 2 to 4 months old Rockland females was examined for estrous regularity, and all clearly irregular or persistently di-

estrous animals were discarded. The selected mice were divided into 2 lots, one fed stock Rockland diet, the other a 7% *Lithospermum* diet mixed with powdered stock Rockland diet, compounded into pellets. During the first week of feeding no vaginal smears were taken; thereafter for 2 months daily smears were made.

The estrus data for the second strain were obtained from females of a group of several hundred C₃H mice which had been placed on a *Lithospermum* diet shortly after weaning. Random samples from the control and experimental C₃H reservoirs were selected for 2-week assays when about 4 months of age. In a second instance, the C₃H females assayed were from a group which had been on a *Lithospermum* diet for about 8 months; and a third sampling was made of the C₃H stand-by group and their normal diet controls when the animals were about 14 months of age (*i.e.*, having been on the *Lithospermum* diet for about 12 months). All of the C₃H females, regardless of age, responded almost uniformly in showing a continuous anestrus, whereas only a partial inhibitory response characterized the Rockland females, confirming observations made earlier.² It is evident that the C₃H strain is considerably more susceptible to the anti-estrous factor in *Lithospermum* than is the Rockland strain. Also, it appears that the cyclic refractoriness described for the latter strain in our earlier paper² is largely absent in the C₃H strain.

Two groups of C₃H females, one having been on *Lithospermum* diet for 8 months, and the other on normal diet for a similar period of time, were assayed for estrus. The *Lithospermum* group had been sampled throughout the 8 months period, and the animals were found to have been in a state of continuous diestrus. The diet of these two groups was reversed: those which had been on *Lithospermum* were placed on normal diet, and conversely. The group which for the preceding 8 months had been in a state of continuous diestrus, immediately returned to relatively normal cyclicity; and the normal animals previously on the stock diet, responded within 96 hours to the *Litho*-

[†] *iso*-androliclactone acetate was selected for test because it is thought to cause an accumulation of gonadotrophins in the anterior pituitary. The compound was supplied through the generosity of Dr. Gregory Pineus of the Worcester Foundation for Experimental Biology.

[§] Supplied by Dr. R. O. Roblin, Jr., of the American Cyanamide Company, Stamford, Conn.



PLATE 1.

Figs. 1 and 4. Thyroid and anterior pituitary from 12-months-old C_3H female mouse which had been in continuous diestrus for 10 months, due to *Lithospermum* feeding.

Figs. 2 and 5. Thyroid and anterior pituitary from 12-months-old C_3H female mouse on normal diet.

Fig. 3. Thyroid from adult C_3H female mouse which had been on 0.1% thiouracil diet for 30 days.

spermum. The immediacy of the return to normal of the former group after being taken off the *Lithospermum* diet indicates that long exposure to the *Lithospermum* factor induces no permanent or irreversible changes within the body.

The weight history of female mice fed on *Lithospermum* diet from weaning, presents some points of interest. Such weight data were obtained each month beginning 5 months after the initiation of the experimental feeding. There was no significant weight difference between experimental and control groups. Cranston,¹ using larger doses—40% *Lithospermum* in the diet—reported that mice undergo an initial weight retardation from which they recover, however, in four months while still under treatment. Our weight data did not cover the age period between 2 and 7 months.

3. *Histology*. Studies were made of various tissues of C_3H mice which had been on 7% *Lithospermum* diet for from 10 to 12 months. No changes were observed in the thyroid epithelium and follicles in animals which for almost a year had been in sustained diestrus due to *Lithospermum* feeding (Fig. 1 and 2). The anterior pituitary, fixed and stained according to a method used earlier,³ appeared to be normal in both control and experimental animals, no marked qualitative or quantitative changes being observed in any of the cell types (Fig. 4 and 5). The uteri appeared somewhat atrophic and were clearly in the anestrus condition. The ovaries were somewhat smaller than normal and showed some follicular atresia. This atresia was similar to but not as marked as that reported for *Lithospermum*-fed Rockland Swiss mice, whose ovaries underwent pronounced atresia following prolonged *Lithospermum* administration.² The mammary tissues of the experimental C_3H animals were rudimentary. It should be noted that control animals used for histological comparison with the experimental ones were sacrificed only when in true diestrus.

4. *The Thyroid*. The possibility that

Lithospermum acts by suppression of the thyroid rather than by affecting the gonadotrophin-producing tissues of the pituitary, seemed to justify further investigation, despite the failure of the pellet-administered thiouracil to show any anti-estrous effect. Accordingly, two sets of mice were fed on diets consisting respectively of 0.1% and 0.5% thiouracil. These diets were fed to C_3H females with a history of normal estrous cyclicity. At these doses there was no disturbance of estrus. An observed body weight loss implies that enough thiouracil was absorbed to exert a systemic effect. Histologically the thyroids of mice so treated with thiouracil showed very marked hyperplasia (Fig. 3). These observations are consistent with the findings of Jones, Delfs, and Foote, who observed no change in the mating behavior of thiouracil-fed rats,⁴ although gestation was often interfered with; but are at variance with those of Mann⁵ who noted lengthening of inter-estrous periods in thiouracil-fed rats.

5. *Distribution of Factor within the Plant*. In all the preceding *Lithospermum* experiments, as well as in those of Cranston, the whole plant was used in the experimental diets. It was of interest to investigate the relative abundance of the anti-estrous factor in the various parts of the plant. Accordingly, dried *Lithospermum* plants were divided into (1) flowers and seeds, (2) roots, (3) leaves, (4) stems. These fractions were pulverized and made up to 15% with normal powdered Rockland mouse diet. Four groups of about 10 female Rockland mice each, on the stock diet, were assayed for estrous regularity for 10 days, then placed on the special diets. Daily estrous assay was continued during a 20-day experimental-diet period. The data are presented in Fig. 6, together with mean body weight curves. It is seen that the factor is practically absent from the stems, and maximally concentrated in the flowers and seeds. Return to normal diet resulted in almost immediate return to normal estrous cyclicity.

⁴ Jones, G. E. S., Delfs, E., and Foote, E. C., *Endocrinology*, 1946, **38**, 337.

⁵ Mann, C. W., *J. Psychol.*, 1945, **20**, 91.

³ Zahl, Paul A., *Z. f. Mikro-Anat. Forsch.*, 1937, **42**, 303.

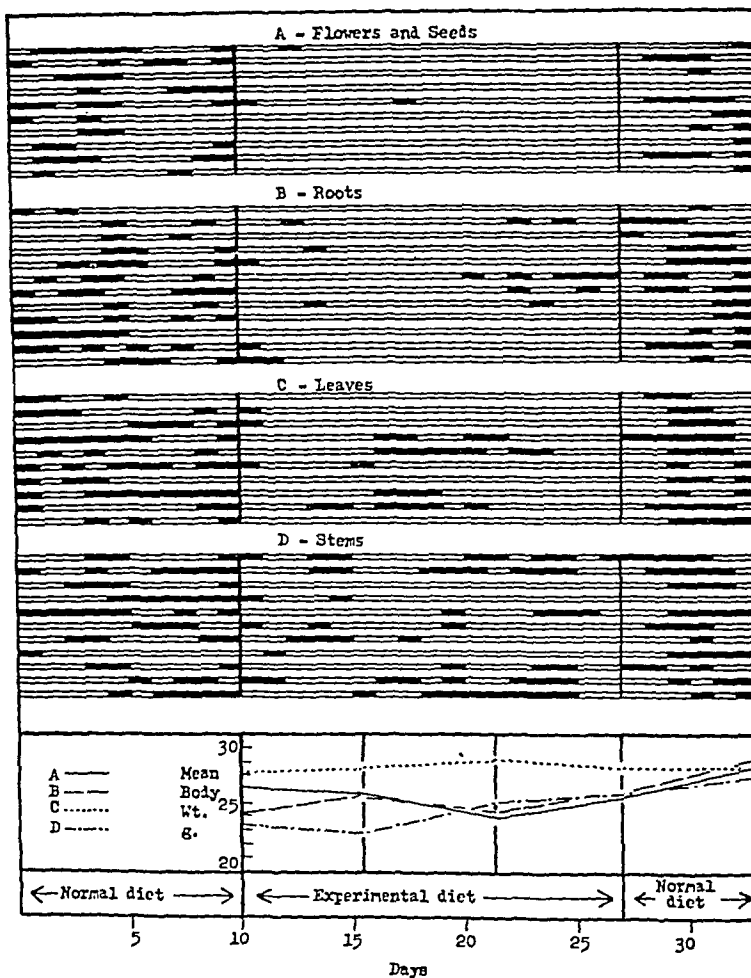


FIG. 6.

Estrous history of mice fed, respectively, on A—Flowers and Seeds; B—Roots; C—Leaves; D—Stems, of the plant *Lithospermum ruderae*. Pulverized, these materials were made up to 15% with normal powdered mouse diet, then compounded into pellets. The experimental feeding was preceded and followed by periods of normal diet feeding. The black horizontal areas indicate duration of estrus. The mean body weight curve for each group of mice is plotted below.

Discussion. It is clearly established that the *Lithospermum* factor, presumably in the water-soluble fraction, produces a pronounced anestrus effect in mice. Our observations that neither the thyroid nor the pituitary exhibit histological changes following protracted *Lithospermum* feeding, do not necessarily militate against the suggestion by Cranston that the effect may be directly on the gonadotrophic elements of the

anterior pituitary. It is certainly possible that an inhibitory effect may occur which is not of sufficient magnitude to elicit observable morphological changes. The fact that mice after 8 months of *Lithospermum*-induced diestrus immediately reestablish normal cyclicity when returned to normal diet, suggests that no permanent changes have occurred in any of the tissues involved. That the effect of *Lithospermum* results from a direct action

on the ovaries has been doubted by Cranston on the grounds that estrone injection immediately reestablishes cyclicity in *Lithospermum*-fed animals. It is possible that the *Lithospermum* effect is a threshold one of just sufficient magnitude to disrupt cyclicity, but insufficient to produce irreversible changes in the tissues involved in estrous cycles. Perhaps the use of a more potent concentrate of the active material would clarify this matter. There is the possibility, too, that the factor interacts with circulating gonadotrophins, although we have neither positive nor negative evidence of such action.

Summary. (1) The anti-estrous factor of *Lithospermum* appears to reside in the neu-

tral water fraction. (2) The anti-estrous factor is most abundant in the flowers and seeds of the dried plant, and almost wholly absent from the stems. (3) Interference with estrus is stopped almost immediately, regardless of the length of treatment, when *Lithospermum* is withdrawn and animals returned to normal diet. (4) The C₃H strain is more sensitive to *Lithospermum* than is the Rockland Swiss strain. (5) The action of *Lithospermum* is unlike that of thiouracil. (6) The factor induces no observable changes in the anterior pituitary, thyroid, suprarenals, or pancreas; some atresia is observed in the ovaries, and some atrophy of the uteri.

16323

In vivo Observations on the Distensibility of the Femoral Venous System.*

ROBERT S. ALEXANDER. (Introduced by Carl J. Wiggers.)

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The marked engorgement of a vein when its central end is occluded tends to create the impression that veins are highly distensible structures. This illusion is probably created by the filling of partially collapsed veins¹ rather than by a true distension of the venous walls, since studies of segments of large veins^{2,3} have revealed a relative lack of distensibility over the physiological range of venous pressures. The question arises as to whether observations on the large veins suited to direct distensibility measurements are necessarily typical of the venous system as a whole. Hochrein and Singer, for example, found the femoral vein considerably more distensible than the inferior vena cava.² Should the smaller veins and venules be

highly distensible, alterations in venous pressure would be of considerable significance in altering the partition of the circulating blood volume between the venous system and other channels of the circulatory system.

Clark⁴ attempted an estimate of the distensibility of the entire venous system of the forearm *in vivo* by employing a plethysmographic method. Her results were difficult to interpret, however, because of the inability to differentiate changes in venous volume from concomitant changes in tissue fluid volume. A more direct approach to this problem in the experimental animal is suggested by the simple observation that if the venous outflow from an organ is watched at the time the outflow pressure is suddenly lowered, the fall in pressure will be observed to produce a transient gush of excess blood. This excess blood obviously represents a volume passively flushed out of the system by

* Supported by a grant from the Life Insurance Research Fund.

¹ Ryder, H. W., Mølle, W. E., and Ferris, E. B., Jr., *J. Clin. Invest.*, 1944, **23**, 333; Duomareo, J., Rimini, R., and Predari, F. N., *Rev. Argentina Cardiol.*, 1946, **12**, 333.

² Hochrein, M., and Singer, B., *Arch. Exp. Path. Pharmacol.*, 1927, **125**, 301.

³ Green, H. D., *Medical Physics*, Otto Glasser, Editor, Year Book Publishers, Chicago, 1944, 213.

⁴ Clark, J. H., *Am. J. Physiol.*, 1933, **105**, 418.

virtue of the reduction in the intravascular distending pressure. In a rigid system such a gush would be absent; in a highly distensible system it should represent a considerable volume. Although it is difficult to delimit the precise anatomical locus from which this excess blood arises, it must be predominantly from the venous system since a pressure change at the point of venous outflow from an organ could have very little effect on the pressures on the arterial side of the circuit. The present study represents an attempt to quantitate this phenomenon in the venous bed of the hind leg of the dog.

Methods. This analysis demanded an accurate method for measuring venous outflow. The simplest procedure is to open the appropriate vein and collect the outflow in a graduated cylinder for an accurately measured time interval. To be adaptable to the present study this method had to be made continuous so that the successive changes in rate of outflow produced by a change in the outflow pressure could be determined. This may be accomplished by determining the increase in volume in the collecting receiver gravimetrically. In preference to the rather cumbersome and frequently inaccurate mechanical devices that have been used for this purpose, we have developed a "strain gauge flowmeter" to obtain a continuous record of the increase in weight of the collecting vessel. This affords a small compact unit which may be placed directly under the receiving vessel and connected to the recording system by light flexible wires of any desired length. The resulting record is a quantitatively accurate graph of the cumulative venous outflow; any excess or deficit in this outflow produced by a change in outflow pressure may be measured directly on the original record.

We employ a gauge manufactured by the Statham Laboratories, Los Angeles, California. This is a self-contained Wheatstone bridge which is accurately balanced to null current when there is no stress applied to the pin actuating the sensitive resistance elements of the bridge. Although designed primarily for the measurement of minute displacements,

these gauges may be used for the direct measurement of stresses in certain ranges. A gauge with a full range of eight ounces is well suited to measuring blood volumes of the order of 100 cc. The output of the gauge is sufficient to directly actuate optically recording galvanometers having periods somewhat less than 0.1 second.

In our application of this device, the outflow receiver (a beaker of 100 cc to 250 cc capacity) is placed on a small aluminum platform 8 x 10 cm. This platform is suspended on 3 points, 2 of which are steel pins pivoting on tapered bearings, and the third is the actuating pin of the strain gauge. The actual displacement of the platform over the full range of the gauge is so slight that frictional resistance in the suspension is of little consequence. To correct for variations in the mechanical advantage of the suspension due to alterations in the precise position of the beaker on the platform, we followed the practice of calibrating each record. This was accomplished by withdrawing an accurately measured volume (usually 20 cc) after each flow measurement and recording the resultant galvanometer deflection before the receiver had been moved from its recording position. Tests of the flow meter revealed almost complete linearity between galvanometer deflection and the volume (weight) in the collecting vessel, the deviation from linearity being 2.5% for the maximum range of the apparatus. By calibrating in the mid-range of each determination, this was reduced to an error of about $\pm 1\%$ and was disregarded.

The dogs were anesthetized with Na barbital (300 mg/kg) and heparinized. In preparing the hind leg, collateral circulation was excluded by severing all connections as high on the hip as possible except for the femoral artery, the femoral vein, the sciatic nerve, and the femur. The sciatic nerve was left intact and carefully protected with saline-soaked cotton to preserve some degree of vasomotor control. The femur was left intact to permit immobilization of the preparation without danger of kinking the blood vessels; collateral flow through the femur itself is negligible.⁵ A cannula inserted dis-

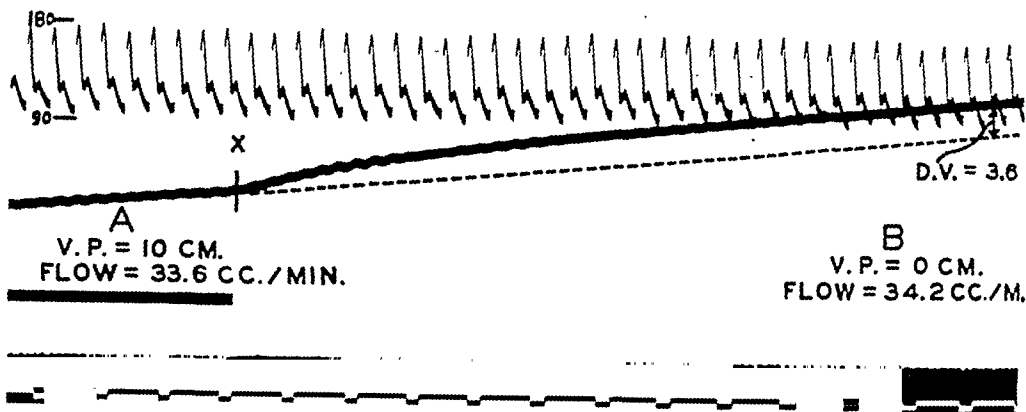


FIG. 1.

Record illustrating method of calculating distensibility volume (D.V.) from flow-meter tracing. At point "X" the venous outflow pressure was lowered from 10 cm to 0 cm. Time signal below indicates 1-second intervals.

tally into the femoral vein was connected by rubber tubing to an outflow orifice. A shunt circuit was provided to permit normal venous drainage of the leg into an external jugular vein between flow determinations as well as to permit return of the blood collected in the flow meter after each observation. The outflow orifice was a short glass tube mounted on a rigid vertical rod to which was attached a centimeter scale. The vertical rod was well lubricated and provided with suitable stops so that the outflow tube could be rapidly shifted from one hydrostatic pressure level to another with a minimum of mechanical jarring. To allow freedom of movement of this outflow tube, the connections with the vein represented a total length of 50 cm. The significant resistance to blood flow which might be introduced by connections of this length was avoided by making them of as large a bore as practical, the rubber tubing having a bore of 6 mm, and cannulae and connecting links having a bore of 4 mm. The outflow pressure as determined by the hydrostatic level of the outflow orifice was read directly in centimeters of blood, zero pressure reference being the approximate level of the vena cava.

The standard recording practice was to allow the flow to equilibrate at the initial

outflow pressure and then record the rate of flow for a period of 10 seconds. The outflow pressure was then quickly shifted to the second level while the recording continued and in general a period of about 20 seconds allowed for equilibration of the flow at this new level. To maintain arterial pressure constant, a compensating reservoir was connected with a carotid artery and elevated to a hydrostatic level of 110 mm Hg. Most dogs of reasonable size would fill the dead space of the reservoir with about 200 cc of blood in equilibrating at this pressure, so that adequate blood was available to compensate for that collected in the flow meter and which was temporarily lost from the circulation. As a check on the arterial pressure perfusing the preparation, optical tracings of the pressure recorded from the opposite femoral artery were superimposed on the flow record.

Experimental Results. The nature of the experimental recordings is illustrated in Fig. 1. The moment of pressure lowering (X) is followed by a transient steep rise which shortly equilibrates at a rate of flow (B) slightly in excess of the initial flow (A). In measuring the records, the assumption was made that at the moment the outflow pressure was lowered the arterial inflow changed to a value equal to the venous flow achieved after final equilibration at B. This assumption is not strictly correct because there will be a

⁵ Green, H. D., Lewis, R. N., Nickerson, N. D., and Heller, A. L., *Am. J. Physiol.*, 1944, 141, 518.

slight lag before pressure-flow relationships equilibrate at the new outflow-pressure level. Analysis of the outflow records with the thought of deriving a correction factor for this error, however, revealed that with changes in outflow pressure of only 10 cm H₂O the error was too small to warrant such an arbitrary correction. The gush of excess blood accompanying the sudden lowering of outflow pressure or what may be termed the "distensibility volume" (DV) may then be measured directly on the graph. For illustrative purposes this measurement is constructed graphically in Fig. 1; in actual practice the records were measured in reference to the time signal marks to correct for any variations in speed of the recording paper.

It is obvious that a comparable measurement may be obtained by the inverse procedure of suddenly elevating the outflow pressure, in which case there would be a momentary deficit in the outflow representing the volume of blood retained by the distension of the system at the higher pressure. Alternate determinations by the two procedures have yielded values in close quantitative agreement. We have selected the first method for routine observations merely be-

cause of the subjective satisfaction of seeing the distensibility volume as a positive increment.

In this fashion the distensibility volumes for 10 cm pressure changes in overlapping intervals for the range of 0 to 30 cm of blood have been determined. Simple addition of these individual values permitted plotting the data in the conventional volume-pressure manner. Two representative curves are shown in Fig. 2. It should be especially noted that the volumes given are those *in excess* of the volume in the system at zero pressure. It is unfortunate that this initial volume cannot be assessed by the present method.

Discussion. As an alternative to distensibility measurements on isolated segments of veins, the present method has a major advantage and an important limitation. Its advantage resides in the fact that it reveals the over-all effect of a change in the central venous pressure on the volume of blood pooled in the peripheral venous channels of the organ under study, and thus the values may be transferred directly to the interpretation of such changes in the circulatory dynamics of the intact animal. In contrast to direct observations on isolated veins, however, these data are of meager assistance in describing the precise physical properties of the system. This latter problem involves far more than the failure to define the exact anatomical limits of the distensible system under study. The pressure change produced at the central end of the femoral vein will not be paralleled by an equal pressure change throughout the femoral venous bed any more than it will produce an equal change in the femoral arterial pressure. On the contrary, it will represent an alteration in the normal venous pressure gradient, the change in this gradient being maximal at the outflow cannula and decreasing progressively as one traces the venous circuit back to the capillaries. It seems highly doubtful that a relatively small change in central venous pressure would significantly alter the pressure in the arterial portion of the capillaries if the arterial pressure head remains constant, a

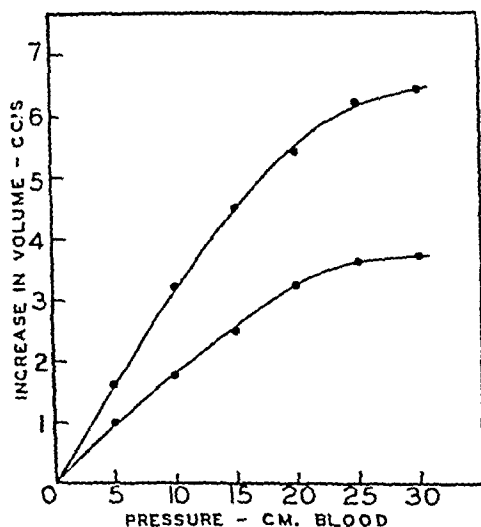


Fig. 2.

Plot of the data obtained from 2 of the dogs in the series showing the increase in volume of the venous bed of the hind leg produced by increases in the outflow pressure in the femoral vein.

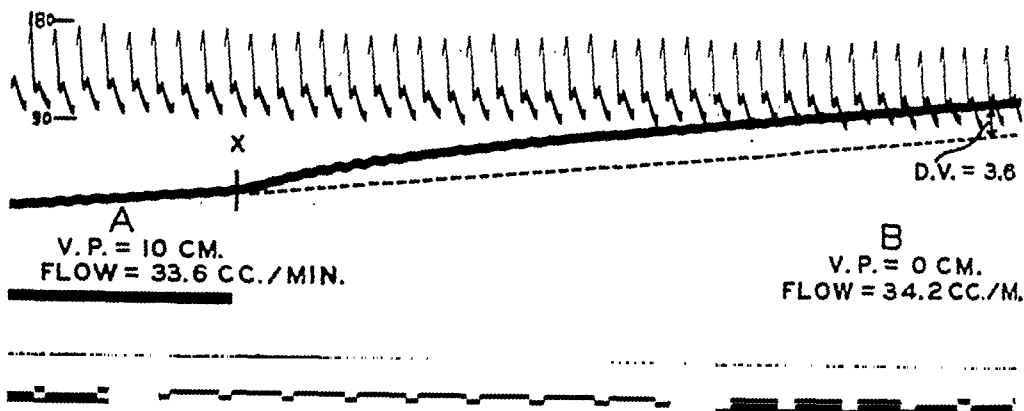


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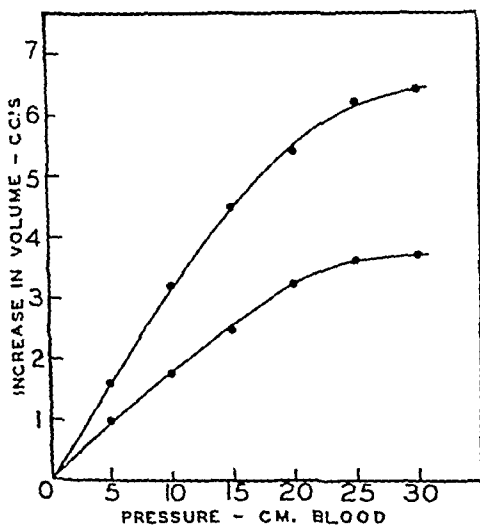


FIG. 2.

Plot of the data obtained from 2 of the dogs in the series showing the increase in volume of the venous bed of the hind leg produced by increases in the outflow pressure in the femoral vein.

relationship which justifies our use of the present method to study venous distensibility. However it also leaves in doubt a knowledge of the precise pressure changes produced in those segments of the venous bed which are yielding the distensibility volume when the central venous pressure is lowered. In addition it must be recognized that measurements of venous distensibility *in situ* are not determined solely by the properties of the vein walls, but may be influenced to some extent by the tension in extra-vascular supporting structures.¹

The results obtained as illustrated in Fig. 2 reveal a volume-pressure relationship that exhibits the same trend as has been found typical of other blood vessels. The relationship is roughly linear over the pressure range of 0 to 20 cm of blood, the range which may be regarded as the range of normal physiological variation. Above this pressure the system appears to be approaching its elastic limit as the curves show a significant flattening in the 20 to 30 cm range. The actual magnitude of the distensibility volumes, moreover, is in accord with inferences derived from measurements on large veins which indicate a relatively low distensibility for the venous system. For a pressure change of 0 to 20 cm we observed an average distensibility volume of only 4.7 cc for the entire venous system in the hind leg of the dog as compared with an actual blood flow that averaged 31.3 cc/min. at an outflow pressure of 0 cm. As central venous pressure rises above this normal range the rigidity of the veins becomes even greater. This indicates that the venous

system of the hind leg serves fairly efficiently as a network of pipes returning the blood from the periphery to the heart.

A complete evaluation of these distensibility characteristics would require information as to the total venous volume of the hind leg. We are not satisfied, however, that valid estimates of the average venous volume of the hind legs of mongrel dogs can be made with information or methods that are at present available. It should also be emphasized that the distensibility measurements reported here deal strictly with passive changes in venous volume without any consideration of changes in the volume of the venous bed which might be produced by venomotor action. There is ample evidence that active changes in the vein walls can occur,⁶ but there is no conclusive evidence to demonstrate their significance *in vivo*.

Summary. The gush of excess blood that appears in the venous outflow from an organ when the outflow pressure is suddenly lowered has been selected as an index of the distensibility of the venous system of that organ. With the aid of a new type of flow meter to quantitate venous outflow, this phenomenon has been studied in innervated hind legs of anesthetized dogs. The results indicate a relatively low distensibility in the venous system of the hind leg of the dog; the volume-pressure data demonstrating a fairly linear relationship in the venous pressure range of 0 to 20 cm of blood, with a trend toward even less distensibility at higher pressures.

⁶ Franklin, K. J., *A Monograph on Veins*, Charles C. Thomas, Springfield, Ill., 1937.

Absence of Prolongation of Pseudopregnancy by Induction of Deciduomata in the Mouse.*

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From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York.

The similarity of the deciduomal reaction to the maternal placenta has prompted several investigations of the possibility that phenomena associated with pregnancy might be found in animals bearing deciduomata. Hammond¹ observed that the mammary development of the pseudopregnant rabbit was not enhanced by the presence of the reaction. Similar results were obtained by Nelson² who found also that surgical removal of the deciduomata did not initiate lactation. More recently, however, Ershoff and Deuel³ have reported that although the presence of deciduomata did not effect the mammary development of the pseudopregnant rat, their presence was associated with a marked delay in the recurrence of estrous vaginal smear. In the normal untreated rat the duration of pseudopregnancy averaged 13.7 days; if deciduomata were induced by traumatizing the uterus on the fourth day, estrus did not recur before the twenty-second day.

The present experiment was performed to determine whether the induction of deciduomata in the mouse would prolong the duration of pseudopregnancy as has been reported in the rat.

Experimental. Twenty young adult female mice of the Swiss albino strain were mated with vasectomized males. Vaginal smears were made on the day following copulation and were taken daily until the appearance

of a completely cornified smear indicated the recurrence of estrus. A control group of 10 mice received no further treatment. In the remaining 10 animals deciduomata were induced by placing longitudinal sutures in the lumina of each uterine horn on the third day after mating. At the end of the experiment these animals were autopsied and the uteri examined grossly to ascertain the presence of deciduomal responses.

In the untreated pseudopregnant mice estrus recurred 11.3 ± 0.68 days after mating; in the group of animals in which deciduomata had been induced, estrus recurred 10.7 ± 0.46 days after mating.

Discussion. The present results show that the presence of deciduomata has no significant effect on the duration of pseudopregnancy in the mouse. The marked prolongation of pseudopregnancy in the rat under similar circumstances³ indicates a decided species difference. The nature of the physiological processes involved is not clear, since in most respects the two species exhibit very similar reproductive phenomena. A clue may be provided, however, in the report of Atkinson⁴ that in the mouse deciduomata induced on the third day of pseudopregnancy persist intact only until the seventh day and then rapidly degenerate. In the rat, on the other hand, Ershoff and Deuel³ report that the deciduomata persist intact until the fourteenth day. Since it has been shown that the persistence of the deciduomal reaction is dependent on the presence of a high level of progesterone,⁴ we may assume that the prolongation of pseudopregnancy in the rat is the result of prolonged luteal activity. The mechanism by which this is effected in the

* Aided by a grant, administered by Dr. Philip E. Smith, from the Rockefeller Foundation, New York.

¹ Hammond, J., *Proc. Royal Soc. B*, 1917, **89**, 534.

² Nelson, W. O., *Anat. Rec.*, 1932, **54** (suppl.), 50.

³ Ershoff, B. H., and Deuel, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 167.

⁴ Atkinson, W. B., *Anat. Rec.*, 1944, **88**, 271.

rat but not in the mouse must remain unexplained at the present time.

Summary. Unlike the rat, the induction of deciduomata does not prolong pseudo-

pregnancy in the mouse. The mechanism responsible for this marked species difference in response to apparently identical physiological conditions is obscure.

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Circulating Antibodies in Vitamin Deficiency States: II. Thiamin and Biotin Deficiencies.

B. B. CARTER AND A. E. AXELROD. (Introduced by Ralph R. Mellon.)

From the Institute of Pathology, Western Pennsylvania Hospital, and Department of Chemistry, University of Pittsburgh, Pittsburgh, Pa.

Introduction. In the first paper of this series,¹ we presented data concerning the effects of pyridoxin, pantothenic acid, and riboflavin deficiencies upon antibody production by the rat in response to human red blood cells as antigenic stimulus. The present paper deals with the effects of thiamin and biotin deficiencies upon antibody production under similar experimental conditions.

Experimental. Male weanling albino rats of the Sprague-Dawley strain were distributed as indicated in Table I. The animals were housed individually in wide-mesh, screen-bottom cages and weighed weekly. Both the thiamin-deficient rats and their controls received a basal diet with the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,² 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *D*-inositol, 0.03; and 2-methyl-1, 4-naphthoquinone, 0.001. For the biotin-deficient rats and their controls, this diet was modified by replacing 60% of the casein with dried egg white. All rats received additional vitamins in the form of a daily pill. Each of the pills given to the 2 control groups supplied the following vitamins: thiamin, 40 γ ; riboflavin, 60 γ ; calcium pantothenate,

200 γ ; pyridoxin, 50 γ ; biotin, 4 γ ; folic acid, 1 γ ; nicotinic acid, 100 γ ; and *p*-aminobenzoic acid, 1mg. For the thiamin- and biotin-deficient groups, the appropriate vitamin was omitted from the pill. The basal diets were fed *ad libitum* to the 2 deficient groups, while the daily food intake of each rat in the control groups was restricted to that consumed during the previous day by its paired member in the corresponding deficiency group.

In Series I, immunization of the thiamin-deficient rats and their controls was begun after 3 weeks on experiment. At this time, the animals were progressively losing weight. Three of the controls and 2 of the deficient rats died during the immunization period. Because of this high mortality, this experiment was repeated exactly in Series II and immunization was instituted after 2 weeks. Although a similar weight loss occurred, no mortality was noted. The biotin-deficient rats and their controls of Series I were immunized after 6 weeks on experiment. At this time, the deficient animals had plateaued in weight and exhibited the typical symptoms of biotin deficiency, *i.e.*, alopecia, dermatitis, blepharitis, and cheilosis.

A 10% suspension of washed, Group O, Rh positive human erythrocytes in normal saline was given intraperitoneally as antigen. An initial dosage of 0.5 ml of the red cell suspension was followed by 2 one cc injections. Inoculations were made on alternate days. Five days after the final injection the

¹ Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 137.

² Jones, J. H., and Foster, C. J., *J. Nutrition*, 1942, **24**, 245.

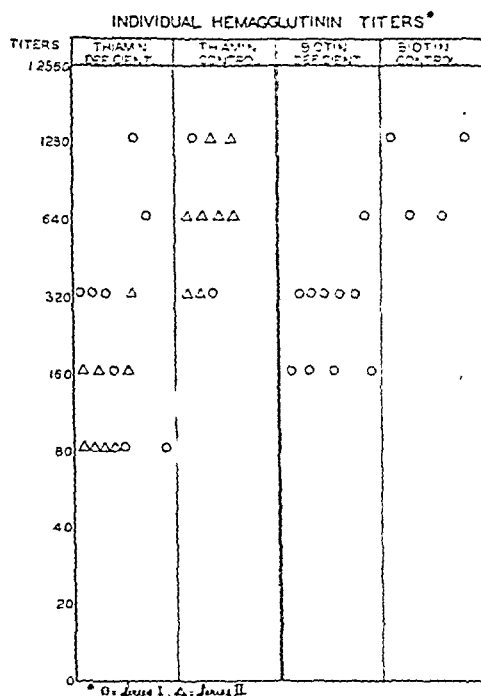
TABLE I.
Summary of Growth and Food Consumption Records.

Series	Group	No. of rats	Body weight*		Daily food consumption*
			Initial	Final†	
I	Thiamin-deficient	8	42	41	2.9
	Thiamin control	2	41	55	2.9
	Biotin-deficient	10	43	176	9.6
	Biotin control	4	44	218	9.6
II	Thiamin-deficient	8	61	64	4.4
	Thiamin control	8	61	70	4.4

* Group avg in g.

† At the time of bleeding.

TABLE II.



rats were bled and the serums tested for agglutinin titer as previously described.¹ Serums of rats from our stock colony possessed no

agglutinins for human Group O, Rh positive red blood cells.

Results. The individual hemagglutinin titers are recorded in Table II. It is evident that the content of circulating antibodies in the thiamin and biotin deficient rats was less than that of the control rats. It is of interest that the decreases noted in the present work are not as marked as those previously observed, in pyridoxin and pantothenic acid deficiencies.¹ It would seem, therefore, that biotin and thiamin are not as critical as pyridoxin and pantothenic acid for optimal antibody response.

Stoerk, Eisen and John³ and Ruchman⁴ failed to observe any influence of a thiamin deficiency upon antibody response in the rat. A direct comparison of our results with those of these workers is difficult because of the variance in experimental procedures.

Summary. (1) Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in thiamin- and biotin-deficient rats. (2) A moderate impairment of antibody response was observed in both deficiencies.

³ Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

⁴ Ruchman, I., *J. Immunol.*, 1946, **53**, 51.

Wound Healing in Rats with Biotin, Pyridoxin, or Riboflavin Deficiencies.

MILTON D. BOSSE AND A. E. AXELROD. (Introduced by Ralph R. Mellon.)

From the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh, Pa.

Retardation of wound healing due to deficiency of vitamin C has long been recognized and the cytologic alterations in wounds consequent to such deficiency have been described. Hunt¹ demonstrated tinctorially that the primary defect of wounds in scorbutic guinea pigs is the immaturity of the intercellular matrix produced by fibroblasts. The role which vitamin deficiencies other than that of vitamin C may play in wound healing has not been adequately studied.

It is the purpose of the present communication to record qualitative and quantitative differences in the healing of 240 wounds in 60 young rats of uniform strain, evenly distributed to include normal controls and those with deficiency of either riboflavin, pyridoxin, or biotin.

Methods: Care of Animals. Weanling, male, albino rats from the Sprague-Dawley strain were divided into 4 groups of 15 each. One group, serving as *controls*, was fed a basal diet of the following composition: sucrose 76, Labco casein ("vitamin-free") 18, salts² 4, and corn oil 2. Each rat received the following vitamins daily in a supplement dish: thiamin HCL 30 γ , pyridoxin HCl 30 γ , riboflavin 30 γ , nicotinic acid 250 γ , calcium pantothenate 100 γ , choline chloride 10 mg, and *i*-inositol 3 mg. The *riboflavin- and pyridoxin-deficient groups* received the same basal diet and supplements as the controls, except for the omission of riboflavin and pyridoxin, respectively. For the *biotin-deficient* group the basal diet was modified by substituting 56% of the casein by powdered egg white. The vitamin supplements were identical with those of the control group.

The animals were housed in individual

cages with wide-mesh screen bottoms and the basal diets were fed *ad libitum*. Each rat was given 2 drops of Abbott's haliver oil (plain) containing 1.3 mg of added d.l. α -tocopherol acetate, weekly, by mouth. The rats were weighed each week.

Production and Characterization of Wounds. After 5 weeks on the respective diets, standard wounds (Series I) were produced on the lateral aspects of each thigh in the following manner: By means of a special stamp a circle 13 mm in diameter was outlined upon the shaved skin with carbol-fuchsin. Under ether anesthesia, by carefully following the outline of this circle with an electric knife, a button of skin extending down to the loose fascia was removed. The intensity of the current was such that the cautery effect did not extend more than one mm beyond the line of excision. No attempt was made to establish aseptic conditions or to cover the wounds. The resulting ulcers, together with a rim of apparently normal skin, were excised under ether anesthesia 3, 6, 14, or 20 days later. The skin edges were approximated with cotton sutures after excision of the ulcers. The excised ulcers were fixed in formalin.

A second series of wounds was made in the same animals 3 weeks following the production of the first series. The procedure was identical with that of the first series, but the site of the wounds was now on both lateral sides of the chest. The lower border of the wounds was over the last rib. Three animals from each group were sacrificed at 3 and at 6 days, respectively. The animals were placed in formaldehyde solution, and the ulcers excised after fixation. Five of the remaining animals in each group were sacrificed at 14, and the other 4, at 20 days, and the ulcers similarly excised.

¹ Hunt, A. H., *Brit. J. Surg.*, 1941, **28**, 436.

² Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

Paraffin sections were prepared from a block which was cut as nearly as possible through the center of the ulcer. These sections were stained with hematoxylin and eosin, a combination of the Van Gieson and the Weigert elastic tissue stains, and the Wilder reticulum stain.

The healing process in each section was studied with regard to the following characteristics:

1. Demarcation and separation of devitalized tissue.
2. The epithelialization of the ulcer.
3. The relative amount of collagen in the scar tissue, as indicated by pink fibers in the Van Gieson, and non-argyrophilic fibers in the reticulum stain.
4. The relative amount of precollagen in the scar tissue, as indicated by yellow fibers in the Van Gieson, and argyrophilic fibers in the reticulum stain.
5. The density of the scar tissue.
6. The vascularity of the scar tissue.
7. The cellular infiltration of the scar tissue.

Results. Observations on wounds of Series I and II are combined since significant differences between the two series were not observed.

General Effects of Dietary Deficiencies. After 5 weeks on the respective diets (the time at which the first wounds were inflicted) the average weights were as follows: biotin-deficient group, 127 g; pyridoxin-deficient group, 82 g; riboflavin-deficient group, 59 g; and control group, 176 g. The average weights of the vitamin-deficient groups remained fairly constant thereafter, whereas that of the control group continued to increase throughout the experiment. Besides the retardation in growth, the animals receiving vitamin-deficient diets developed the symptoms characteristic of the respective deficiencies. These symptoms were more severe during the healing of the second wounds than they were while the first wounds were healing.

Gross Observations of Wounds. At no time was there gross evidence of infection. All wounds remained dry and clean. The

ulcers of the control and biotin-deficient groups appeared to heal equally rapidly, while the healing of the ulcers of the pyridoxin- and riboflavin-deficient groups was strikingly delayed. There was also a considerable difference in the thickness of the skin of the rats, detectable by palpation. The skin of the control and biotin-deficient groups was moderately thick, in contrast to the thin skin of the other two groups (confirmed by microscopic measurement).

Toward the end of the first week following the production of the wounds, the ulcers of the control and biotin-deficient groups were definitely smaller than those of the other two groups. Since microscopic section at this time showed very little epithelial or connective tissue growth, the difference in the size of the ulcers could be explained only on the basis of a difference in the contraction of the skin about the ulcers.

The ulcers of the control and biotin-deficient groups were healed at 14 days and those of the pyridoxin- and riboflavin-deficient groups, at 20 days.

Microscopic Observations of Wounds (see Fig. 1). An early reaction in the wounds is a narrow zone of leukocytic infiltration which forms at the junction of devitalized tissue (from the cautery effect of the cutting current) with viable tissue. This leukocytic zone of demarcation extends from the epidermis to the base of the ulcer. Epithelium regenerating from the surface and from sweat glands and hair-follicles adjacent to the leukocytic zone grows immediately subjacent to the latter. Later a cleft develops in the leukocytic zone which marks the beginning separation of this devitalized tissue. Because of the similarity of this process to the sequestration of necrotic bone, it will hereafter be referred to as sequestration. The separating, necrotic, burned tissue becomes an integral component of the eschar, together with dried serum, condensed fibrin, blood, and cellular exudate.

Biotin-deficient group. During the first 2 weeks there is some retardation in collagen production and in the density of the granulation tissue, while the amount of precollagen



FIG. 1.

Photomicrographs of representative areas of 14-day-old wounds stained with a combination of the Van Gieson and the Weigert elastic tissue stains, $\times 130$. A is from a control rat; B, from a riboflavin-deficient rat; C, from a pyridoxin deficient rat; and D, from a biotin-deficient rat.

and the vascularity are greater than is seen in the normal animal. At 20 days the scar is identical with that of the control.

Pyridoxin-deficient group. Initially there is retardation of leukocytic reaction and in the development of granulation tissue. Epithelialization is delayed throughout. Although terminally these ulcers are epithelialized as well as the control ulcers, the scars contain less collagen and a considerable amount of precollagen. The latter is absent

at this time in the control and biotin-deficient groups. The scar tissue is less dense, more vascular, and more cellular than that of the control group. During the course of healing there is retardation of four to seven days in the evolution of the lesion, as compared with that of the control group.

Riboflavin-deficient group. There is delay not only in the evolution of the granulation tissue, but also in the epithelialization of the ulcer. Granulation tissue is tardy in appear-

ance and terminally it contains a decreased amount of collagen with considerable admixture of precollagen. The scar is less dense than that of the control group, but more dense than that of the pyridoxin-deficient group. The vascularity and cellularity are increased.

Discussion. Disturbance in the production of collagen is generally considered characteristic of scurvy, but is also known to occur in hypoproteinemia. The results obtained in the present experiments indicate that a similar disturbance is associated with deficiency of biotin, pyridoxin, or riboflavin in rats. However, the retardation of collagen production in these vitamin-deficiencies is less pronounced and less persistent than in scurvy.

The pyridoxin- and riboflavin-deficient groups exhibit, in addition, delayed contraction of the wound as observed grossly, and tardy exudation; the latter resulting in delayed sequestration and production of granulation tissue. The residual increased vascularity and correlative decreased density of the scars of the pyridoxin- and riboflavin-deficient groups are further indications of retardation in wound healing which are logically associated with the terminally decreased collagen and increased precollagen.

The wound of biotin-deficient animals exhibits a somewhat diminished density and decreased collagen content of the scar. There is no retardation of exudation or of granulation tissue production. Terminally the scar is indistinguishable from that of a normal animal.

The role which inanition plays in the retardation of wound healing is difficult to assess. It may or may not be coincidence that the greatest retardation in wound healing occurs in animals where inanition is also maximal—in the riboflavin- and pyridoxin-deficient groups. However, while the retardation in wound healing is greater in pyridoxin-deficient rats than in the riboflavin-deficient animals, inanition is greater in the latter than in the former.

Summary. The process of wound healing has been observed in rats exhibiting severe deficiency of biotin, pyridoxin, or riboflavin. Marked impairment of rate and quality of healing was noted in the pyridoxin- and riboflavin-deficient groups. The biotin-deficient group showed only mild delay in healing.

We are indebted to Merck and Company, Inc., for the B vitamins used in these studies.

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Peptidases in Human Serum.*

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(Introduced by J. S. L. Browne.)

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Although the existence of proteolytic enzymes in blood serum and leucocytes has long been recognized, no definitive work has been done in this field until recent years (cf. review of the early literature, Opie¹).

With the development of the use of synthetic di- and tri-peptides as substrates, a

quantitative microtitration technique was devised for the determination of peptidase hydrolysis (Grassmann and Heyde,² Abderhalden and Hanson,³ Maschmann,⁴ Fruton,⁵

² Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1929, **183**, 32.

³ Abderhalden, E., and Hanson, H., *Fermentforsch.*, 1937, **15**, 382.

⁴ Maschmann, E., *Biochem. Z.*, 1941, **308**, 359.

⁵ Fruton, J. S., *J. Biol. Chem.*, 1946, **166**, 721.

* This study was aided by a grant from the John and Mary Markle Foundation.

¹ Opie, E. L., *Physiol. Rev.*, 1922, **2**, 552.

TABLE I.
Hydrolysis of LGG, GL, and GG by Normal Human Serum.
(The hydrolysis of LGG is most rapid and considerably activated by cobalt ions.)

Substrate .05 M	cc human serum per cc test sol.	Metal ion	Hydrolysis * % per hour
LGG	.4	— (avg of 5)	7.3
	.4	Mg (.01 M)	5.0
	.4	Mn (.001)	5.5
	.4	Co (.001)	9.6
GL	.4	— (avg of 4)	2.3
	.4	Mg (.01 M)	1.2
	.4	Mn (.001)	3.4
	.4	Co (.001)	2.5
GG	.4	—	0.0
	.4	Co (.001 M)	3.0

* % per hour of the hydrolysis expected on the complete splitting of one peptide linkage.

and Holman *et al.*⁶). Except for some preliminary observations on pathological human serum with l-leucylglycylglycine as substrate (Grassmann and Heyde⁷) the method has found no clinical application thus far.

A study was undertaken to investigate proteolytic activity of the human serum in clinical conditions associated with ageing. As a preliminary step, however, it was necessary to study this function in normal control subjects and under various conditions involving leucocytosis and tissue proliferation.

Method. Blood was obtained from non-fasting subjects by venous puncture. Serum was prepared by centrifugation.

The tri-peptide l-leucylglycylglycine (LGG) and the di-peptides glycyl-l-leucine (GL) and glycylglycine (GG) were used.[†] In the activation studies the method described by Maschmann⁴ was followed, in which 0.01 M cobalt, 0.01 M manganese or 0.1 M magnesium were added as sulphates. The reaction was carried out in a water bath at 39°C in 2.0 ml volumetric flasks containing 0.05 mM/cc of the substrate. The pH was maintained near pH 7.8 with 0.01 M phosphate or veronal buffer, both of which were found to be equally effective. 0.01 ml of toluene was

added to each tube as preservative. In the preliminary experiments on LGG, GG and GL, 0.4 cc of serum per ml of reaction mixture were used. With cobalt as activator, the concentration of serum was reduced to 0.2 cc per ml of test solution in the studies on LGG. The experiments on the 11 normal control subjects and the pathological cases were carried out on LGG with this serum concentration. The rate of proteolytic hydrolysis was followed in triplicate according to the titration method of Grassmann and Heyde.² Four readings were taken over a period of 7 hours, following a zero time determination. The results were corrected for serum and substrate controls which were run simultaneously. The rate of activity per hour was calculated from the slope of the zero order plot of the per cent hydrolysis versus time (per cent hydrolysis equals per cent of the hydrolysis expected on the complete splitting of one peptide linkage).

Subjects. Eleven members of the staff (5 males and 6 females) ranging in age from 24 to 47 years were studied as normal controls. Investigations were carried out on 10 subjects suffering from various pathological conditions. These included dental extraction, postpartum infections, fractures, aleukemic leukemia, cancer and non-malignant tumor.

Results. The results are presented in Tables I and II. In the serum of 11 normal control subjects the rate of hydrolysis ranged from 6.1 to 7.8% per hour with an average of $7.2 \pm 0.78\%$.

⁶ Holman, H. R., White, A., and Fruton, J. S., *Proc. Soc. Exp. Biol. and Med.*, 1947, 65, 196.

⁷ Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1930, 188, 69.

[†] These peptides were obtained through the courtesy of Dr. M. S. Dunn at the University of California in Los Angeles.

TABLE II.
 Action of Serum Peptidases in Various Abnormal Conditions.*

Diagnosis	Condition	Age	Sex	Hydrolysis % per hr
Vulvar carcinoma, epilepsy	Sed. rate 39	39	F	9.4
Cervical carcinoma	" " 30	47	F	5.2
" " "	" " 52	52	F	10.9
Non-malignant tumor	" " 49	46	F	6.4
Aleukemic leukemia	WBC: 6,500/cu mm Lym.: 4,680/cu mm High sed. rate	47	M	7.4
Postpartum pneumonia	6th day postpartum	23	F	11.0
" infection	10th " "			7.6
	Penicillin treatment	29	F	9.6
	10th day postpartum			
Leg fracture	Fever 4th day after fracture	58	M	13.0
" "	Day of fracture	62	M	12.6
Lumbar and arm fracture	Day of fracture	35	F	14.6
	6th day			8.4
Dental extraction	1 day after	26	F	5.5

* The reaction mixture consisted of 0.05 M LGG, 0.001 M cobalt, 0.01 M veronal or phosphate buffer; 0.2 cc of serum were used per cc of reaction mixture.

The manner of compiling the data is illustrated in Fig. 1.

Discussion. The present investigation shows that there exists in human serum a cobalt-activatable LGG-splitting peptidase. The rate of hydrolysis is uniform in healthy adult subjects and there are considerable deviations from this normal range in various

pathological conditions. The results obtained in this study (Table I) agree with the data of Grassmann and Heyde⁴ on human serum. Fruton⁵ calculated from this data a value of 0.035% hydrolysis per minute (K° LGG) for 0.1 ml of serum per ml of test solution. Since we found direct proportionality between serum concentration and rate of reaction,[‡] our data yields a corresponding K° LGG of 0.030 ± 0.01 .

From our limited number of cases more detailed conclusions can be drawn only with great reservation. The fact that the rate of hydrolysis was highest in cases of puerperal infection and fractures, and decreased with the progress of healing, suggests further studies along the lines of tissue reaction and repair. This is also indicated by the observations of Zamecnik *et al.*⁶ who reported a rise of serum peptidases in animals following burns.

Summary. The rate of hydrolysis of l-leucylglycylglycine by the cobalt activatable peptidase in human serum shows little variation in normal adults, but is increased in certain pathological states, particularly in fractures and postpartum infections.

‡ Unpublished experiments.

⁶ Zamecnik, P. C., Stephenson, M. L., and Cope, O., *J. Biol. Chem.*, 1945, 158, 139.

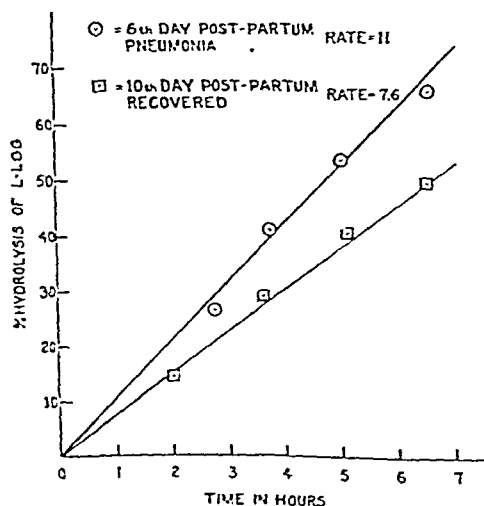


FIG. 1

Hydrolysis of LGG by serum from a case of postpartum pneumonia. It may be seen that the kinetics of the reaction are of a zero order.

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⁶ Holman, H. R., White, A., and Fruton, J. S., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 196.

⁷ Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1930, **188**, 69.

[†] These peptides were obtained through the courtesy of Dr. M. S. Dunn at the University of California in Los Angeles.

stir up the precipitate by forcing the alcohol wash solution over it in a sharp stream. The last 1 to 2 ml of the wash solution should be used to wash down the sides of the centrifuge tube.

Discussion. It will be noted that in the preparation of the urine sample, as given above, a 1:10 dilution of urine is prepared and that the phosphate has not been removed. We have found only negligible differences in comparative values obtained whether or not phosphate is removed at this dilution. An advantage offered by the extreme sensitivity of this method is that samples of very low sodium content are immediately recognized at the first step in the precipitation; *i.e.*, a minute amount of precipitate forms. In such cases, it usually suffices to repeat the precipitation using the preparation as recommended by Butler and Tuthill in which the urine is not diluted during the removal of protein and phosphate.

In the determination of serum sodium, a 1 ml aliquot of the solution of ashed sample containing the equivalent of 0.1 ml of serum is used. Here, again, the great dilution eliminates the necessity for the removal of phosphate.

Recoveries of sodium added to urines of known value are shown in Table I. Comparison of results obtained with the proposed method and that of Butler and Tuthill are presented in Table II.

The color characteristics of the water solution of the sodium uranyl zinc acetate complex were determined using both a Beckman Model DU Spectrophotometer and a Coleman Model 11A Spectrophotometer. Absorption maxima were obtained at 430 $m\mu$ on both instruments. Plots of concentration *vs* density do not give a straight line at this wave length. However, the hyperbolic curve obtained is smooth and highly reproducible.

The yellow color of the dissolved complex is stable for at least 4 hours. The intensity

TABLE I.
Recovery of Sodium Added to Urine Samples.

Meq. sodium added	Meq. sodium recovered	% recovered
.0118	.0113	95.8
.0118	.0116	98.3
.0059	.0055	93.3
.0059	.0057	96.6
.0020	.0020	100.0
.0020	.0021	105.0

TABLE II.
Comparison of Results with Colorimetric and Gravimetric⁴ Procedures.

Gravimetric—Meq./liter	Colorimetric—Meq./liter
36.0	36.0
69.0	68.8
28.6	30.0
63.6	65.0
82.3	81.4
89.0	87.5

varies with temperature, and unknown solutions must be read at the same temperature at which the standard curve was prepared.

In explanation of the sensitivity obtained by this method, it may be suggested that the intrinsic errors in weighing and possible imperceptible small leakage through the pores of the sintered glass filter are additive factors in the gravimetric procedure, which are eliminated in the procedure described.

Summary. A photometric method is described based on the gravimetric procedure of Butler and Tuthill in which the precipitated sodium zinc uranyl acetate is dissolved in water, giving a yellow color which is read with a suitable filter against a standard curve at 430 $m\mu$. Phosphates in the normal urine were found to exert negligible interference. Recoveries and comparison values show the method to be more rapid and at least of equivalent accuracy with the gravimetric procedure.

I wish to express my appreciation to Professor C. J. Farmer and Dr. Edith B. Farnsworth for their aid in carrying out this study, and to Miss Lora Belle Hughes who performed most of the technical work.

A Photometric Adaptation of the Zinc Uranyl Acetate Method for Sodium.

JACOB S. KRAKUSIN.* (Introduced by C. J. Farmer.)

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Several colorimetric methods suitable for use with biological material have been proposed for the determination of sodium.^{1,2,3} The disadvantage of these in routine biochemical analysis varies from a lack of the necessary sensitivity^{1,2,3} to interference from chromogenic materials usually encountered.³

The proposed method is a modification of that of Butler and Tuthill.⁴ All reagents used are described in that method. The precautions and stoichiometric relations are carefully preserved while the tedious weighing and washing procedures have been eliminated. In addition, a greater sensitivity is obtained which makes possible the use of a smaller volume of sample and the analysis of fluids with very low sodium content.

The procedure will be described for urine. Serum and other fluids may be prepared for analysis according to Peters and Van Slyke⁵ with appropriate dilution to bring the solutions into the range of the standard curve.

Method. Add to 5 ml of urine in a 50 ml volumetric flask, 5 ml of 20% trichloroacetic acid. Mix, and dilute with water to the mark. After again thoroughly mixing, filter out any precipitated protein, if present, using a dry Whatman No. 40 or 42 filter paper and discarding the first 5 ml of filtrate.

To 10 ml of uranyl zinc acetate reagent in a 15 ml graduated pyrex centrifuge tube, add dropwise 1 ml of the urine filtrate (or

prepared sample of serum, etc.) with constant stirring. Mechanical stirring is desirable though not imperative. Allow the mixture to stand for 30 to 45 minutes with occasional stirring. Remove the stirring rod, washing down with 3 ml of reagent as it is being removed. Cap the tubes (IEC No. 580 rubber caps) and centrifuge at 2000 r.p.m. for 5 to 10 minutes. Aspirate off the supernatant fluid as completely as possible. Wash the precipitate with 95% ethyl alcohol saturated with sodium zinc uranyl acetate, cap and centrifuge as above. Aspirate the supernatant and repeat the washing and aspiration. After drying the tubes in an oven or water bath at 60° to 70°C for 5 to 10 minutes, add 8 ml of hot water (60°C) to dissolve the precipitate. Cool to room temperature, make up to the 10 ml mark with water and mix. Bring the temperature to 25°C in a water bath and read at 430 m μ in a spectrophotometer or with a suitable blue filter in any photoelectric colorimeter. The zero density setting is made using a reagent blank of 1 ml water treated and washed in the same manner as the sample. The concentration of the unknown is read from a standard curve prepared with sodium chloride solutions containing 0.002 to 0.05 meq sodium/ml.

Precautions. 1. Stirring of the solution should be continued for at least 1 minute after the precipitate appears. Incomplete stirring will give erroneously low results.

2. Aspiration of the supernatant fluid in the centrifuge tube is preferable to decantation because the granular quality of the precipitate is such that it could easily be lost through rough handling. It is obvious that as much as possible of the supernatant fluid should be removed with each aspiration. This is best done if a drawn out capillary tube is used as an aspirator.

3. In the washing procedure it is best to

* Aided in part, by a grant from the United States Public Health Service.

¹ Salit, P. W., *J. Biol. Chem.*, 1932, **96**, 959.

² Arnold, A. E., and Pray, A. R., *Ind. and Eng. Chem. (Anal. Ed.)*, 1943, **15**, 294.

³ Bradbury, J. T., *J. Lab. Clin. Med.*, 1946, **31**, 1257.

⁴ Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

⁵ Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Baltimore, Williams and Wilkins, 1932, p. 733.

TABLE I.
Bacteriostatic Effect of Chloromycetin on 20
Strains of Tubercle Bacilli.

Strain No.	Type	Concentration in μg per ml which completely inhibited growth	
		Without serum	With serum
H37Rv	Human	12.5	12.5
H37RvR*	"	12.5	12.5
1	"	12.5	12.5
1R	"	12.5	25.0
11	"	12.5	12.5
12	"	12.5	12.5
15	"	3.12	12.5
15R	"	3.12	12.5
18	"	1.56	12.5
21	"	6.25	12.5
24	"	12.5	12.5
24R	"	6.25	25.0
69	"	12.5	12.5
69R	"	12.5	12.5
97	"	6.25	12.5
97R	"	6.25	12.5
100	"	12.5	12.5
100R	"	12.5	12.5
111	"	12.5	12.5
48	Bovine	No growth	25.0

* R indicates a streptomycin-resistant strain.

between 6.25 and 12.5 μg per ml to completely inhibit growth. The bacteriostatic activity of chloromycetin for these same 12 strains in the presence of serum was not significantly reduced. The remaining 7 strains which, in the absence of serum were inhibited in their growth to a greater degree by chloromycetin, were, in the presence of serum, as resistant as the other 12. Whether the decrease in sensitivity to chloromycetin shown by the above 7 strains in the presence of serum was due to the inactivation of chloromycetin by serum or was merely a reflection of the stimulation of growth of tubercle bacilli which occurs in the presence of serum is not discernible from these data. While the figures given in Table I represent the least amount of chloromycetin which under the conditions of the test completely inhibited growth, partial retardation of growth was usually noted in one-half or one-fourth this concentration. In any event, chloromycetin *in vitro* is considerably less bacteriostatic for virulent human type tubercle bacilli than is either streptomycin or para-aminosalicylic acid since with these latter substances most strains of tubercle bacilli are completely in-

TABLE II.
Bacteriostatic Action of Chloromycetin in Com-
bination with PAS and Streptomycin.

	Cone. in μg per ml which com- pletely inhibited growth
Chloromycetin	12.5
PAS	0.78 ^s
Streptomycin	0.78
Chloromycetin + PAS	1.56
" + Streptomycin	1.56
" + PAS + Streptomycin	1.56

hibited in their growth by less than one or 2 μg per ml.^{7,8}

Since the bacteriostatic activity of many chemotherapeutic agents such as the sulfonamides, sulfones and PAS is markedly affected by the number of organisms used in the test, the bacteriostatic action of chloromycetin was tested using larger numbers of tubercle bacilli. Since a 10-fold increase in the amount of inoculum used in the test resulted in only a 4-fold decrease in bacteriostatic activity, the action of the drug, in contrast to para-aminosalicylic acid⁷ is apparently not appreciably influenced by the number of organisms present.

In spite of the relatively low tuberculo-static action of chloromycetin, it was thought advisable to determine *in vitro* the effect of combining this substance with para-aminosalicylic acid and streptomycin. Table II shows the results of these tests. It is apparent that chloromycetin contributed nothing to the bacteriostatic action of such combinations since in no case was the effect greater than that obtained with either para-aminosalicylic or streptomycin alone. There was in fact consistently a slight reduction in bacteriostatic action when chloromycetin was included in the medium. Since this difference is well within the experimental error of the method, no great significance at present can be attached to it.

The initial experiments with chloromycetin to determine its therapeutic effectiveness *in vivo* were conducted with intravenously tubercularized mice by treating subcutaneous-

^s Raleigh, Gordon W., and Youmans, Guy P., *J. Inf. Dis.*, 1948, in press.

Tuberculostatic Action of Chloromycetin *In vitro* and *In vivo*.*

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The Evanston Hospital, Evanston, Ill.

Ehrlich, Bartz, Smith, Joslyn and Burkholder¹ reported the isolation of a *Streptomyces* sp. which produced a new antibiotic which they named Chloromycetin. This antibiotic was highly bacteriostatic *in vitro* for a variety of bacteria, but only moderately bacteriostatic for the virulent H37Rv strain of *M. tuberculosis*. Chloromycetin showed considerable activity against *Rickettsia prowazekii* in tests using chick embryos. Smadel and Jackson² have also reported that chloromycetin was an effective chemotherapeutic agent experimentally against a variety of Rickettsiae. In a recent, more detailed report Smith, Joslyn, Gruhitz, McLean, Penner and Ehrlich³ have further described the properties of chloromycetin, including data on toxicity and chemotherapeutic activity. The *in vitro* tests of the bacteriostatic activity of chloromycetin for *M. tuberculosis* var. *hominis* (H37Rv) reported in the above publications were done in this laboratory. This work has now been extended to include *in vitro* tests on other strains of virulent human-type tubercle bacilli and the effect of chloromycetin administered subcutaneously and orally on experimental murine tuberculosis.

Methods. The *in vitro* bacteriostatic tests with chloromycetin⁴ were performed by the technic previously reported^{1,5,6} and the re-

sults recorded in terms of the least amount of chloromycetin which would completely inhibit the subsurface growth of 0.01 mg of tubercle bacilli per ml of synthetic medium. Duplicate tests were conducted in the same medium to which had been added enough sterile beef serum to make a final concentration of 10.0%. Nineteen strains of virulent human-type tubercle bacilli were employed, including 7 which were resistant to more than 1000.0 µg of streptomycin per ml. One bovine strain was also used. Similar *in vitro* tests were conducted in which chloromycetin was employed in combination with streptomycin and para-aminosalicylic acid (PAS).

The chemotherapeutic action of chloromycetin *in vivo* was determined by using mice infected intravenously with 0.1 mg of the H37Rv strain of *M. tuberculosis*. The technic employed in these tests and the evaluation of the results of the tests were in every respect similar to those previously reported employing streptomycin and PAS,^{6,7} with the exception that chloromycetin, when administered subcutaneously to mice, was dissolved in 20% propylene glycol water solution and the experiments were terminated at the end of 21 days instead of 28 days.

Results. The results of the *in vitro* bacteriostatic tests with and without serum using the 19 human type strains and the one bovine strain are shown in Table I. There was as much as an 8-fold variation in the sensitivity of these strains to chloromycetin when serum was omitted from the medium. Since twofold dilutions were used, the majority of the strains (12 out of 19) required

* Work aided by a research grant from Parke, Davis & Company, Detroit, Mich.

¹ Ehrlich, John, Bartz, Quentin R., Smith, Robert M., Joslyn, Dwight A., and Burkholder, Paul R., *Science*, 1947, **106**, 417.

² Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

³ Smith, Robert M., Joslyn, Dwight A., Gruhitz, Oswald M., McLean, Wm. I., Jr., Penner, Mildred A., and Ehrlich, John, *J. Bact.*, 1948, **55**, 425.

⁴ Crystalline chloromycetin obtained from Parke, Davis Company, Detroit, Mich.

⁵ Young, Guy P., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 119.

⁶ Young, Guy P., and Doub, Leonard, *Am. Rev. Tuberc.*, 1946, **54**, 287.

⁷ Young, Guy P., Raleigh, Gordon W., and Young, Anne S., *J. Bact.*, 1947, **54**, 409.

⁸ Young, Guy P., and McCarter, John C., *Am. Rev. Tuberc.*, 1946, **52**, 432.

If this one determination represented the average serum concentration of chloromycetin during the course of treatment one would expect the slight borderline therapeutic effect which was noted since this concentration of chloromycetin, while it does not completely inhibit the growth of H37Rv strain *in vitro*, will partially retard the multiplication of the bacilli.

Summary and Conclusions. The antibiotic chloromycetin has been found to be only moderately bacteriostatic for virulent human type tubercle bacilli *in vitro* as compared with streptomycin or para-aminosalicylic acid. The majority of 19 human type strains studied were completely inhibited in their

growth by between 6.25 and 12.5 micrograms or more of the drug in the presence of serum. One bovine strain was equally sensitive. This degree of bacteriostatic activity was not markedly affected by the number of tubercle bacilli present nor was the bacteriostatic activity of para-aminosalicylic acid or streptomycin enhanced by the addition of chloromycetin.

When administered subcutaneously, chloromycetin has been shown to be ineffective, whereas, when admixed with the diet in concentrations of 0.5 and 0.25%, it was slightly effective for the suppression of experimental murine tuberculosis.

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Subtenolin. An Antibiotic from *Bacillus subtilis*. I. Bacteriologic Properties.

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(Introduced by Henry Tauber.)

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In the course of a search for antibiotic agents in this laboratory, an organism was isolated in February, 1945, that produced a substance showing antibiotic activity for certain gram-positive and gram-negative bacteria. The organism was isolated from nutrient agar plates that had been seeded with *Escherichia coli* and *Staphylococcus aureus*, respectively, and inoculated with material from dusty surfaces in the laboratory. It was identified as *Bacillus subtilis*.* Because of its microbic source and strong enolic properties¹ the name, subtenolin, has been given to the antagonist. The bacteriologic properties of the antibiotic are the subject of this

report; its isolation from the harvest and its chemical properties are described in the succeeding report.¹

Production of Subtenolin. Media containing peptones and other complex organic enrichments were found to be very poor for the production of subtenolin. A medium containing *dl*-alanine produced the highest yields and least destruction. With subtenolin, as with subtilin,² strong stimulatory action by manganese was observed. Production was slightly stimulated by copper.

The following medium was adopted for use in the production of subtenolin:

* Acknowledgment is made to N. R. Smith of the U. S. Department of Agriculture, Beltsville, Maryland, for the final identification of the organism.

¹ Howell, S. F., and Tauber, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 432.

² Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, **4**, 297.

TABLE III.

Results of the Mice Infected with *M. tuberculosis* (H37Rv) and Treated with Chloromycetin in the Diet.

Compound	% of compound in diet	No. mice	% mortality	Avg wt loss or gain in g	Amt gross pulmonary tuberculosis	Type of lesion
Chloromycetin	0.5	20	0.0	-4.1	3.5+	P & NE
	0.25	20	30.0	-3.3	3.84+	P & NE
	0.125	20	65.0	-3.85	3.85+	NE
Para- <i>amino</i> -salicylic acid	1.0	20	0.0	+1.6	2.5+	P
Controls		20	75.0	-4.8	4.0+	NE

1+ — 0-10% involvement of lung with tuberculosis

2+ — 10-25% " " " " "

3+ — 25-50% " " " " "

4+ — 50-100% " " " " "

P — Proliferative lesions

NE — Necrotic-exudative lesions

ly with 5 mg of chloromycetin per day dissolved in 20% propylene glycol. This was divided in 2 daily doses of 2.5 mg each given approximately 8 hours apart. This total daily dosage was selected on the basis of the known toxicity of chloromycetin administered by this route³ and approached closely the maximal tolerated dose. These experiments failed to demonstrate any suppressive effect on the tuberculous process. Furthermore, marked reaction developed at the site of injection with swelling, edema and eventually ulceration.

When it became apparent that chloromycetin was well absorbed from the gastro-intestinal tract, experiments with intravenously tubercularized mice were conducted in which the compound was administered with the diet. Table III details the results of a typical experiment. Included in this table in addition to the untreated controls is a group of animals treated with 1.0% para-*amino*-salicylic acid. From the data given in the table, it is readily apparent that there was a relationship between the per cent mortality of the treated mice and the concentration of chloromycetin administered. Those mice which received 0.5% chloromycetin in the diet survived the 21-day period of treatment. The group which received 0.25% showed 30% mortality and the group which received 0.125%, a 65% mortality. The last figure is very similar to the mortality figure for the untreated control group. There was no significant difference between the amount of weight lost by any of the chloromycetin treated animals and the untreated controls; in contrast, the para-

aminosalicylic acid treated animals showed a slight increase in average weight. The data given for the amount of gross pulmonary tuberculosis demonstrable at the end of the experimental period in the various groups correlates with the weight response. On the basis of these findings one might question the significance of the mortality figures for the groups of animals receiving the 0.5% and the 0.25% chloromycetin. However, upon microscopic examination, whereas the total amount of involvement of lung substance was large, those animals treated with 0.5% and 0.25% chloromycetin showed a significantly higher proportion of proliferative type lesions as compared with the controls. These findings, as has previously been determined,⁸ indicate that the treated animals had a slower evolution of the disease process. The histopathological findings in the mice which received the smallest amount of chloromycetin (0.125%) did not differ from the untreated controls. It can be stated with some confidence that chloromycetin administered orally to intravenously tubercularized mice in a concentration of 0.5%, and possibly 0.25%, exerted a slight suppressive effect upon the disease process. Before the animals which received 0.5% were sacrificed at the termination of the experiment they were bled by cardiac puncture approximately 2 hours after being fed and the small blood samples obtained were pooled. The serum thus obtained was tested for its content of chloromycetin[†] and was found to be 7.0 µg per ml.

† Performed by Dwight A. Joslyn, Parke, Davis & Company, Detroit, Mich.

TABLE I.
The Antibacterial Spectrum of Subtenolin.

	Minimal inhibiting quantity (mg)	
	5 hr	22 hr
Preparation No. 165, 300 units/mg*		
1. <i>Staphylococcus aureus</i> , FDA 209P	.004	0.55
2. " " MB 111	.004	n.i.†
3. " " 30393	.004	n.i.
4. " " H	.0065	n.i.
5. " albus, 30389	.0023	0.47
6. <i>Streptococcus</i> , sp. (enterococcus group VD)	.023	1.25
7. <i>Bacillus anthracis</i> , ATCC 8705	2.0	n.i.
8. <i>Micrococcus conglomeratus</i> , MB 78	.024	n.i.
9. <i>Escherichia coli</i> , ATCC 7011	.014	1.67
10. <i>Salmonella enteritidis</i>	.015	n.i.
11. <i>Eberthella typhosa</i> , MB 59	.019	1.0
12. <i>Salmonella schottmuelleri</i> , M	.047	2.0
13. " typhimurium, ATCC 9148	.078	n.i.
14. <i>Serratia marcescens</i> , D	.16	n.i.
15. <i>Aerobacter aerogenes</i> , ATCC 8308	.16	n.i.
16. <i>Klebsiella pneumoniae</i> , ATCC 9997	.71	n.i.
17. <i>Pasteurella pestis</i> , A 1122	i.g.‡	0.055
Preparation No. 176, 550 units/mg*		
1. <i>Staphylococcus aureus</i> , FDA 209P	.0021	0.28
2. <i>Streptococcus pyogenes</i> , C203	.54	2.7
3. <i>Diplococcus pneumoniae</i> , type III	2.72	n.i.
4. <i>Clostridium novyi</i> , AMS	i.g.	0.55
5. " perfringens, AMS	i.g.	0.55
6. " fallax, AMS	i.g.	0.91
7. " tertium, AMS	i.g.	0.91
8. " histolyticum, AMS	i.g.	1.1
9. " ordensis maligni, AMS	i.g.	1.4
10. <i>Bacillus subtilis</i> (subtenolin producing strain)	n.i.	n.i.
11. " R	n.i.	n.i.
12. <i>Micrococcus lysodeikticus</i> , MB 79	.17	n.i.
13. <i>Pasteurella</i> , sp., MB 90	i.g.	0.041
14. <i>Eberthella typhosa</i> , PCI 412	.0065	0.303
15. <i>Escherichia coli</i> , P6	.018	0.68
16. " MB 60	.064	2.7
17. " VD	.026	n.i.
18. <i>Pseudomonas aeruginosa</i>	2.7	n.i.
19. <i>Neisseria gonorrhoeae</i> , strain 1	i.g.	1.1
20. " strain 2	i.g.	1.1
Preparation No. 189, 850 units/mg*		
1. <i>Staphylococcus aureus</i> , FDA 209P	.0014	0.17
2. <i>Brucella abortus</i> , USDA 2610	i.g.	n.i.
3. " melitensis, USDA K1957	i.g.	n.i.
4. " suis, USDA, S452	i.g.	n.i.
5. <i>Salmonella paratyphi</i> , ATCC 9150	n.i.	n.i.

* One unit is defined as the minimal concentration of subtenolin completely inhibiting the growth of *Staphylococcus aureus*, FDA 209P in total volume of one ml after five hours incubation period. In the determination of the antibacterial spectrum, a reference solution was prepared by dissolving subtenolin in distilled water to give a concentration of 15,000 units/ml.

† n.i. = no inhibition.

‡ i.g. = insufficient growth.

by strains of *Bacillus subtilis* include subtilin,² bacitracin,⁵ bacillin,⁶ eumycin,⁷ and

subtilysin, a lytic principle obtained by Vallée⁸ from culture filtrates. In addition, coli-

⁴ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

⁵ Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

⁶ Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1946, **51**, 363.

⁷ Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

⁸ Vallée, M., *C. R. Soc. biol.*, 1945, **139**, 148.

	%
DL-alanine	0.3
Potassium dihydrogen phosphate	0.5
Magnesium citrate • 14H ₂ O	0.25
Magnesium sulfate • 7H ₂ O	0.05
Glycerol	2.0
Manganous sulfate • 5H ₂ O	0.0004
Cupric sulfate • 5H ₂ O	0.00005
Water, distilled, to volume	

The medium was adjusted to pH 6.8-7.1, dispensed 300 ml per one-gallon bottle, and autoclaved at 15 lb for 15 minutes. The bottles were inoculated with 0.5 ml of a standardized spore suspension (tube No. 0.5 McFarland barium sulfate standard) that had been prepared from discrete colonies. To obtain maximum surface exposure, the bottles were incubated in a horizontal position at 36°C for 3 days. Subtenolin activity reached its peak when growth was greatest (3 to 4 days) and remained constant until one or 2 days later when antibiotic activity steadily decreased with increased alkalinity of the culture medium.

Lyophilization of the spores in 25% gelatin proved to be the best method for maintaining the strain of *Bacillus subtilis* for consistent maximum production of subtenolin. By preparing a new batch of lyophilized spores at least every 3 months, the level of subtenolin production was held fairly constant throughout the period of this work.

In practice, the inoculum was prepared by reconstituting the contents of an ampoule of lyophilized spores and preparing tenfold dilutions in saline for plating on FDA agar.³ After incubation for 4 to 7 days at 30°C, individual rough colonies were selected and suspended in 0.85% NaCl solution. This suspension was standardized and inoculated in the medium described above. Harvesting of the culture was done by screening through several layers of gauze and cotton to remove most of the bacterial pellicle.

General Properties of Subtenolin. Subtenolin diffuses very readily through agar, producing large and clearly defined zones with *Staphylococcus aureus* as the test organism. Many preparations showed the presence of isolated, resistant colonies within the clear

area of inhibition.

The antibacterial activity of subtenolin is variously reduced by complex organic substances. In whole, defibrinated rabbit blood there is almost complete loss of activity in 2 hours. Several peptones also reduced the antibacterial effect of subtenolin. However, rabbit or horse serum, FDA broth,³ and heart infusion broth had no effect when tested by the agar streak method.

Toxicity studies by intraperitoneal injection of mice weighing 17 to 23 g showed the LD₅₀ to be 30 to 60 mg (1000 units/mg). Considerable subtenolin activity was found in the urine 15 minutes after injection and, depending upon the dosage, continued 4 to 10 hours. With a dose of 50 to 60 mg (1000 units mg), subtenolin continued to be excreted for 10 hours after intraperitoneal injection. On the average, 30 to 50% of the administered dose was found in the urine.

Assay Methods and Antibacterial Spectrum. The serial dilution method using FDA broth³ was the procedure adopted for subtenolin assay. Since there was reduction of subtenolin activity after overnight incubation but none after 5 hours, the tests were read at 5 hours and again at 22 hours. The endpoint selected was the highest dilution of a standard subtenolin solution showing complete inhibition of the test organism. For the more fastidious organisms, heart infusion broth containing 10% rabbit serum and 1% dextrose was used. This medium reduced subtenolin activity 50 to 80% after overnight incubation although no reduction in activity was observed after 5 hours incubation.

The antibacterial spectrum of subtenolin is summarized in Table I. The most susceptible organisms were: *Staphylococcus aureus*, *Staphylococcus albus*, *Eberthella typhosa*, *Escherichia coli*, *Salmonella enteritidis*, *Micrococcus conglomeratus*, and *Salmonella schottmuelleri*. Two strains of *Pasteurella* showed marked susceptibility to the antibiotic upon overnight incubation. The growth of *Mycobacterium tuberculosis* H37Rv in Dubos medium⁴ was partially inhibited by a concentration of 2000 units/ml of subtenolin.

Differentiation of Subtenolin from Other Antagonists. Antibiotic substances produced

³ Federal Register, Oct. 17, 1946, 11, 12128.

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	5 hr	22 hr
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4. " suis, USDA, 8452	i.g.	n.i.
5. <i>Salmonella paratyphi</i> , ATCC 9150	n.i.	n.i.

* One unit is defined as the minimal concentration of subtenolin completely inhibiting the growth of *Staphylococcus aureus*, FDA 209P in total volume of one ml after five hours incubation period. In the determination of the antibacterial spectrum, a reference solution was prepared by dissolving subtenolin in distilled water to give a concentration of 15,000 units/ml.

† n.i. = no inhibition.

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⁶ Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1946, **51**, 363.

⁷ Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

⁸ Vallée, M., *C. R. Soc. biol.*, 1945, **139**, 148.

statin, apparently produced by an unidentified member of the genus *Bacillus*, has been reported.⁹ Subtenolin differs from these antagonists of similar origin as described below.

Subtilin. Subtilin is extracted from the cellular material of the pellicle and is light-sensitive. Subtenolin is extracted from the culture medium and is not light-sensitive.

Bacitracin. Bacitracin does not inhibit *Escherichia coli*, *Aerobacter aerogenes* nor *Eberthella typhosa*, but these organisms are susceptible to the action of subtenolin. Unlike bacitracin, subtenolin is relatively inactive against *Streptococcus pyogenes*, pneumococcus and clostridia.

Bacillin. Subtenolin and bacillin display a loss of activity in the presence of certain complex organic substances (although not to the same degree), and their production is stimulated by manganese. On the other hand, carbohydrates promote the production of bacillin but interfere with the production of subtenolin. Also, carbohydrates are required for demonstrating the anticoli activity of bacillin but not of subtenolin. Bacillin is not excreted in the urine of mice[†] but subtenolin activity has been found in the urine of mice after intraperitoneal injections. *Streptococcus pyogenes* and *Diplococcus pneumoniae* Type III are relatively susceptible to bacillin but resistant to the action of

subtenolin. In comparative studies with bacillin, subtenolin was found to be 5 times as active against *Staphylococcus aureus* 209 P on FDA agar.

Eumycin. Eumycin was reported to have no activity against *Eberthella typhosa* and colon bacilli and only slight activity against staphylococci. Subtenolin definitely inhibited these organisms, especially staphylococci.

Subtilysin. Subtilysin shows activity for various clostridia and none for staphylococci. Subtenolin shows the opposites of these activities.

Colistatin. The presence of glucose in the medium is required for colistatin production, but this sugar is not favorable for subtenolin production. Contrary to subtenolin, colistatin inhibits the pneumococcus.

Summary and Conclusions. 1. An antibiotic, tentatively designated subtenolin, was isolated from a strain of *Bacillus subtilis*.

2. Although the antagonist displays its greatest activity against staphylococci, the growth of certain gram-positive as well as gram-negative organisms is inhibited.

3. The growth of *Mycobacterium tuberculosis* is partially inhibited by subtenolin.

4. Subtenolin activity has been detected in urines recovered from intraperitoneally inoculated mice.

5. Acute toxicity studies show that the LD₅₀ of subtenolin for mice inoculated intraperitoneally is 30 to 60 mg (30,000-60,000 units).

⁹ Gause, G. F., *Science*, 1946, **101**, 289.

[†] Personal communication from H. B. Woodruff of Merek and Company.

16331

Subtenolin. An Antibiotic from *Bacillus subtilis*. II. Isolation and Chemical Properties.

STACEY F. HOWELL AND HENRY TAUBER.

From the Biochemistry Section, Venereal Disease Research Laboratory, U. S. Marine Hospital, Staten Island, New York.

The method of isolation and the chemical properties of subtenolin, an antibiotic produced by a strain of *Bacillus subtilis*, are described in this paper. The method used in

the isolation of the antibiotic is based on its ability to be concentrated by adsorption on activated carbon, its solubility in methyl alcohol, its insolubility in butanone, and its

stability when dried from a methyl alcohol solution. Subtenolin has very interesting chemical properties. It is extremely sensitive to drying in the presence of water. However, in aqueous solution, it is very stable to heat. This antibiotic has strong enolic properties. The bacteriological study of the antibiotic is described in the preceding paper.¹

Experimental. Isolation of the Antibiotic. Ten liter portions of the medium, the composition of which has been given in the preceding article,¹ were processed each time. The pH of the broth was usually between 6.7 and 6.8. The pellicles were removed by screening through several layers of cheesecloth, then by centrifuging in a Sharples supercentrifuge. Forty grams of charcoal (Darco G-60) were added per liter of culture fluid. The mixture was shaken during a period of 20 minutes and filtered on Buchner funnels. The carbon cake was washed with 2 liters of water and transferred to a 5-liter bottle. The antibiotic was eluted with 2 liters of methyl alcohol by shaking for 30 minutes. After filtration the eluate was concentrated *in vacuo* at 40°C to 30 ml.

The concentrated eluate was poured into 1500 ml of butanone (contained in a beaker) and stirred with a glass rod until solidification of the antibiotic took place. The precipitate was washed twice with 100 ml portions of butanone and twice with 100 ml portions of ether. The antibiotic was extracted from the precipitate with one 80 ml and two 30 ml portions of methyl alcohol. The mixture was filtered to remove undissolved material. The white residue on the filter paper was washed with two 30 ml portions of methyl alcohol. Upon solution in water this material showed insignificant activity. The combined methyl alcohol extracts were evaporated to 8 ml *in vacuo* at 45°C. A small amount of inactive material which separated at this point was removed by centrifuging and discarded. The methyl alcohol solution was evaporated to dryness *in vacuo* at 45°C. The dried preparation was stored *in vacuo* over CaCl₂.

The yield of subtenolin was about 1.0 g

of light yellow powder per 10 liters of medium. Preparations with 1000 to 1600 dilution units per milligram, representing 25 to 50% recovery, were obtained. It is important to note that when solutions of the antibiotic containing more than traces of water are evaporated to dryness, the antibiotic is largely destroyed. Apparently most of the water present in the concentrated eluate was removed by the butanone treatment without inactivating the antibiotic.

Chemical Properties of Subtenolin. The antibiotic is very soluble in water, in ethylene glycol, in 95% methyl alcohol, and in many organic solvents containing a small amount of water. It is insoluble in acetone, butanone, ether, and 95% ethyl alcohol. It is not precipitated by acids or alkalis. Subtenolin is very heat stable. No loss of activity occurs when a solution in water is autoclaved for 15 minutes at 15 lb pressure. No destruction takes place at pH 2.0 during 18 hours at room temperature. The dried preparations may be kept for months in a vacuum without loss of potency. Subtenolin dialyzes readily through cellophane membranes and is somewhat hygroscopic in the dry state. The antibiotic in neutral solution gives a strong "peroxidase" test with p-phenylenediamine and hydrogen peroxide (purple color).² It gives a strong enol test,² a typical Molisch test, a blue color with ninhydrin, and a dark brown color with ferric chloride. It forms a crystalline hydrazone with 2,4-dinitro-phenyl-hydrazone in alcoholic solution. When a saturated solution of picric acid in ethyl alcohol is added to a concentrated aqueous solution of subtenolin, the solution turns brown and a crystalline substance is obtained in the form of rosettes. This crystalline material is inactive. Subtenolin reduces permanganate, iodine, and bromine in the cold, and Benedict's qualitative sugar reagent and ammoniacal silver nitrate solution on boiling. It reduces Shaffer-Somogyi's sugar reagent one-half as much as penicillin G and about one-sixth as much as glucose when two milligrams of each of the substances were used

¹ Hirschhorn, H. N., Bucca, M. A., and Thayer, J. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67: 429.

² Feigl, F., *Qualitative Analysis by Spot Tests*, Nordemann Publishing Company, Inc., New York, 1939.

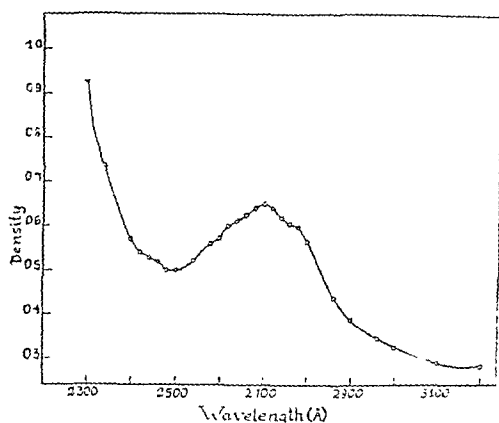


FIG. 1.

Adsorption spectrum of subtenolin in the ultra-violet region. One cc contained 0.4 mg of the antibiotic in distilled water. The pH was 6.52.

per test solution. The purified product contains 51.4% carbon, 7.00% hydrogen, 7.88% nitrogen, and 0.81% sulfur.

A solution of subtenolin in normal hydrochloric acid becomes rose colored upon boiling. Upon the addition of an excess of alkali the solution turns yellow but returns to rose color when reacidified. Subtenolin is destroyed by hydrogen sulfide but not by cysteine. Pepsin (Parke, Davis & Company, 1:3000), trypsin (Difco Laboratories, 1:250), polidase-S (Schwarz Laboratories), and tyrosinase (1830 units per ml)³ have no effect on the antibiotic.

The antibiotic exhibits a striking absorption of light in the ultraviolet region of the spectrum (see Fig. 1). The curve has a high sharp peak at 2700 Å and shows a minimum absorption at 2500 Å. The Beckman spectrophotometer was used.

Discussion and Summary. It may be seen from Table I that subtenolin is quite different chemically from other antibiotics such as streptomycin, penicillin G, gramicidin, and bacitracin. Subtenolin, bacitracin, bacillin, subtilin, and eumycin are products of various strains of *B. subtilis*. Gramicidin⁴ and bacitracin⁵ appear to be peptides. Subtilin, which

³ Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R., *J. Am. Chem. Soc.*, 1944, **66**, 514.

⁴ Hotchkiss, R. D., *Adv. Enzymol.*, 1944, **4**, 153.

⁵ Johnson, B. A., Anker, H., and Meloney, F. L., *Science*, 1945, **102**, 376.

TABLE I. Some Chemical Properties of Subtenolin and Other Antibiotics.

	Subtenolin, 1000 u/mg	Penicillin G Sodium (Com'l Solvents Corp. 1622 u/mg)	Streptomycin (Merck & Co. CaCl ₂ Complex Crystalline)	Gramicidin (Wallenstein Co.)	Bacitracin (Com'l Solvents Corp., 34 u/mg)
Molisch's Test	+	+	—	—	—
Benedict's Qualitative Sugar Test	+	+	—	—	—
Millon's Test	—	—	—	—	+
Hopkins Cole Test	—	—	—	—	—
Biotin Test	—	—	—	—	—
Ninhydrin Test	Yellow on boiling	—	Light blue	Intense purple	Purple
Trichloroacetic acid (10%)	Blue	—	"	Intense violet	"
Ferric Chloride	—	—	—	Blue	"
Enol Test	Dark brown	—	—	Heavy precipitate	Turbid
Indicator Test	+	—	Yellow	—	Yellow
<i>p</i> -Phenylenediamine Test	Rose (turns yellow in alkaline sol.)	Same as control	Same as control	Same as control	Same as control
In neutral sol.	Dark purple	"	"	"	"
In acid sol.	Yellow	"	"	"	"

In all tests 5 mg of the respective antibiotics were used except in the *p*-phenylenediamine test in which 0.5 mg of each of the antibiotics was used. The *p*-phenylenediamine test was carried out by adding 0.5 ml M phosphate buffer pH 7.0, 0.5 ml of 1% *p*-phenylenediamine in water, and 0.5 ml 3% hydrogen peroxide to 0.1 ml of the respective antibiotic solutions. When this test was carried out in acid medium 2 M acetic acid was substituted for the phosphate solution.

is extracted from the pellicles of a particular strain of *B. subtilis*, is of unknown chemical composition.⁶ Eumycin is prepared by acid precipitation of the medium.⁷ The chemical relationship between bacillin and subtenolin has not been ascertained since data pertaining to the chemical nature of bacillin is not available for comparison.⁸

⁶ Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.*, 1947, **15**, 1.

⁷ Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

⁸ Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1946, **51**, 371.

Subtenolin has a low molecular weight. Its chemical and physical properties indicate that it contains a resonating double bond, phenolic groups, a very active enolic group, and an aromatic aldehyde radical. This antibiotic gives typical color reactions such as the Molisch and enol reaction. These tests, and the indicator test shown in Table I, may be used in its identification. Although the antibiotic has not been obtained in a pure state, its chemical and antibiotic properties, particularly its stability in aqueous solution or when dry, make it an interesting object for further studies.

16332

Respiratory Arrest in Rabbits Exposed to Hypoxia after Dibenamine.*

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The observations here reported were made as part of a systematic elucidation of the effect of alteration of function of the autonomic nervous system on the response of the intact animal to hypoxia.

Rabbits underwent early and sudden respiratory arrest on exposure to hypoxia after receiving Dibenamine (N, N-dibenzyl-β-chloroethylamine) in ethyl alcohol and propylene glycol aa by vein. Control animals underwent such arrest very rarely even under more prolonged similar exposure to hypoxia.

Procedure. Normal rabbits (weight 1.5 to 4 kg) under intravenous pentothal anesthesia were subjected to tracheotomy. Pro-

caine hydrochloride 2% was infiltrated through the margins of the incision and skin closure around the cannula was made with skin clips.

The rabbits were restrained in the supine, head low position on a table offering a slope of 15° from the horizontal in order to aid the venous return. After recovery (minimum 30 min.) from the general anesthesia the inspiratory ventilation volume on room air was measured by spirometer. This was made possible by an hydraulic flutter valve† interposed between the tracheal cannula and the spirometer. The dead space of the system was 3 to 5 cc. This compares favorably with the dead space of the eliminated respiratory tract above the cannula in the rabbit.

Measurements were then continued as the animal was abruptly shifted to an atmosphere of 5% oxygen in helium for a period of 5 minutes. After one or a series of such control

* Work done in partial fulfillment of Contract No. W33-038ac-18469 for U. S. Air Forces Aero Medical Laboratory by Boston University School of Medicine. Grateful acknowledgment is made for the following supplies used: To Abbott Laboratories for Pentothal Sodium, to Bristol Laboratories for Procaine Hydrochloride, to Givaudan-Delawanna, Inc., for Dibenamine.

† Work done in partial fulfillment of the requirements for the degree of Master of Arts, Boston University Graduate School.

‡ A modification of the type pictured on page 206, Jackson, D. E., *Experimental Pharmacology and Materia Medica*, 2nd Edition, 1939, C. V. Mosby Co., St. Louis, Mo.

TABLE I.
Occurrence of Respiratory Arrest on Exposure to Circa 5% O₂ for 5 Minutes.

Treatment	No. of rabbits showing no arrest	No. of rabbits showing arrest
Normal	49	2
Dibenamine in Propylene Glycol and Ethyl Alcohol $\bar{a}\bar{a}$ 12-24 mg/kg*	2	20
Dibenamine in 50% Alcohol 12-24 mg/kg	1	4
Dibenamine in 50% Propylene Glycol 12-24 mg/kg	1	5
Propylene Glycol 50% 2 cc	6	0
Alcohol 50% average 0.5 cc	6	0
Propylene Glycol and Absolute Alcohol $\bar{a}\bar{a}$ average 0.5 cc	6	0

* By Chi square test the probability of chance distribution of 29 positive in 33 as opposed to 2 positive in 49 is less than 1 in 10,000.

observations on the effect of hypoxia, Dibenamine (at a concentration of 50 mg/cc in the chosen medium)[§] was administered in marginal vein of ear in calculated dose over a period of one minute. Usually the primary dosage of Dibenamine was 12 mg/kg.

After a variable delay the ventilation volume on room air was measured and the animal was again presented with the hypoxic atmosphere for 5 minutes. If respiratory arrest occurred artificial ventilation was established after 1 to 2 minutes of apnea, by means of human expired air through the tracheal cannula. Successive periods of hypoxia were rarely at intervals less than 20 minutes.

Gas mixtures were made in large rubber bag of some 1500 liter capacity and analyzed for oxygen content repeatedly through the day's experiments. Maximum variation of different days' mixtures was 4.0-5.5% oxygen (mean 5% \pm 0.5).

Observations. Table I records the incidence of respiratory arrest in rabbits exposed to hypoxia for 5-minute periods after various medications.

Normal Controls. The 2 cases of respiratory arrest in 49 normals showed arrest only once each in several trials. Time required for arrest to occur was 230 and 240 seconds respectively. It was repeatedly shown that 5-minute periods of hypoxia did not predispose to respiratory arrest in later 5-minute periods of exposure (11 animals up to 9 trials

each over periods up to 6 hours).

Dibenamine: Incidence. In the total series of 38 animals given Dibenamine 5 died within 10 minutes of the intravenous injection of 12 mg/kg. Twenty-three animals showed arrest during hypoxia after the first 12 mg of the drug. Six animals required a second dose at the end of an hour or more before arrest would result from hypoxia. The remaining 4 animals never showed respiratory arrest.

The pattern of respiratory arrest after Dibenamine was bizarre. The rise of ventilation volume at the beginning of the period of hypoxia (average 59% rise) differed little from that of the control hypoxic condition (average 72% rise). However, after an average exposure of 158 seconds (in 44 trials, range 30 to 300 seconds) the respiration became more labored, followed abruptly by an apparently tonic contraction of the diaphragm, accompanied by twitching movements of the abdomen. Simultaneously the tidal volume fell to zero and the tonic contraction faded out. Thereafter no respiratory movements appeared in most animals though one or two gave a feeble gasp reflex.

Duration of susceptibility to respiratory arrest. In animals tested serially to determine the time course of susceptibility to respiratory arrest on exposure to hypoxia, arrest could be elicited at the earliest in most animals between 20 and 40 minutes (mean 33 minutes) after the administration of the drug. Susceptibility endured throughout the period of test (up to 4 hours) in a number of animals. In others the ability to compensate for hypoxia returned, after as little as

[§] Media used included Propylene glycol and absolute alcohol $\bar{a}\bar{a}$ most commonly. Propylene glycol and water $\bar{a}\bar{a}$ or absolute alcohol and water $\bar{a}\bar{a}$ on other occasions.

48 minutes following administration of Dibenamine.

Controls receiving solvents without Dibenamine. See Table I.

Discussion. That the susceptibility to respiratory arrest is not dependent on some residual effect of the pentothal was demonstrated by the occurrence of respiratory arrest in cats under the same conditions except that cyclopropane was utilized during the preparatory period. The pilot experiments in the cat also showed that the susceptibility to arrest is not species specific in the rabbit alone.

That the helium was not an active factor in the elicitation of respiratory arrest was shown in animals tested on nitrogen and oxygen (5%). Five minutes on this mixture never caused arrest in several trials in each of 11 animals tested before Dibenamine. It was at least as potent in producing respiratory arrest after Dibenamine.

That the phenomenon observed was a true respiratory arrest was shown by the demise of 8 animals allowed to go untreated in the apnea which occurred during hypoxia after Dibenamine. No animal ever recovered spontaneously from this state of arrest. On resuscitation spontaneous respiration was re-established promptly after a minute or more

of artificial ventilation with human expired air.

As to the mechanism of action of Dibenamine in inducing respiratory arrest during hypoxia little evidence is available. Pilot experiments have shown that bilateral vagotomy, with its removal of inhibitory sensory discharge does not prevent the occurrence of arrest on hypoxia after Dibenamine (3 animals). Three experiments suggested that Dibenamine does reverse the normal pressor response to hypoxia in rabbits as in other species. However, the fall of blood pressure is inconstant and respiratory arrest has been noted unaccompanied by a depressor response. Acapnia seems an unlikely cause of the apnea, since the hyperventilation normally seen in hypoxia of this grade is at least as great as that after Dibenamine. Finally, a direct depressant action of Dibenamine on the respiratory center seems unlikely, since the center still responds to the carotid body under hypoxia by inducing hyperventilation.

Summary. Rabbits given Dibenamine 12 to 24 mg/kg in propylene glycol or ethyl alcohol by vein show sudden respiratory arrest within 3 minutes of exposure to hypoxia (circa 5% O₂ in helium).

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Results of Heterophile Antibody Agglutination and Kahn Tests in Patients with Viral Hepatitis.*

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(Introduced by H. A. Reimann.)

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Attempts to develop a *specific* immunologic test for viral hepatitis have been unsuccessful, up to the present, although positive re-

sults have been described in certain serologic reactions in this disease.^{1,2,3} Eaton *et al.*² reported that 34% of a group of pa-

* This work was conducted under the auspices of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

¹ Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, P., and Schwenker, F. F., *Am. J. Hyg.*, 1944, **39**, 337; *Am. J. Hyg.*, 1944, **40**, 35.

tients in the acute phase of hepatitis developed an heterophile antibody which agglutinates sheep erythrocytes in a titer of 1:40 or greater. The titer of this antibody rarely rises above 1:160, and may be differentiated from that appearing in patients with infectious mononucleosis by its absorbability on boiled guinea pig kidney and human liver.

It has also been reported that a certain number of patients with viral hepatitis develop transient falsely positive tests for syphilis,⁴ and as many as 20% of one group of patients were reported to have falsely positive Kahn and Wassermann tests during the course of acute hepatitis.⁵

Such a substantial percentage of positive serologic tests, occurring during the course of hepatitis, might constitute a problem in diagnosis. Certain similarities between hepatitis and infectious mononucleosis, such as the presence of cervical lymphadenopathy and lymphocytosis as well as the frequent occurrence of evidence of hepatic dysfunction in the latter disease, make differential diagnosis more difficult.

During recent years, experiments conducted under the auspices of the Army Epidemiological Board on the transmission of infectious hepatitis to volunteers have made it possible to study certain serologic reactions in these patients. In addition, a recent opportunity to survey the serologic response of a large number of patients with hepatitis in an American Army hospital has provided certain data. It is the purpose of this paper to report on the results of the heterophile antibody agglutination, Kahn, and cold agglutinin tests in these two groups of patients.

Method and Materials. Subjects. The patients are divided into 2 groups. *Group I:* 30 previously healthy male volunteers, ranging in age from 19 to 29 years, contracted

infectious hepatitis experimentally, following inoculation or ingestion of material known to contain infectious hepatitis virus. The strain of virus used in these experiments has been previously described.⁶ *Group II:* 478 subjects were patients in an Army hospital in Germany from June 1947 to February 1948. They had contracted acute hepatitis during the performance of duty. It is quite probable that many of these cases were examples of homologous serum jaundice, but in view of the fact that there are no means at present for differentiating infectious hepatitis from serum hepatitis clinically, the cases have all been classified as viral hepatitis. The diagnosis of hepatitis was made in both groups on the basis of characteristic symptoms and signs, accompanied by consistent deflection of appropriate tests of hepatic function. All patients in this report had clinical jaundice.

In Group I, the heterophile antibody agglutination, Kahn, and cold agglutinin tests were determined before experimental inoculation. All were negative. These subjects were tested again during the 2nd, 3rd and 4th weeks of disease, and sera with positive reactions were retested at weekly intervals until negative.

In Group II, these tests were performed in various stages of infectious hepatitis, ranging from the 1st to the 30th week. Numerous serial determinations were made, and the majority of tests were done during the first 8 weeks of disease. Sera with positive reactions were retested at weekly intervals until negative.

Results. Heterophile antibody agglutination tests. Technique of test. The tests were carried out according to the method of Stuart.⁷ Sera with a titer of 1:56 were absorbed on boiled guinea pig kidney and retested.

The results of the determinations in 508 patients represented in Groups I and II are

² Eaton, M. D., Murphy, W. D., and Hanford, V. L., *J. Exp. Med.*, 1944, **79**, 539.

³ Olitski, L., and Bernkopf, H., *J. Infect. Dis.*, 1945, **77**, 60.

⁴ Waelsh, J. H., *Brit. Med. J.*, 1946, March 9, 353.

⁵ Kuzell, W. C., and Puccinelli, V., *Bull. U. S. Army Med. Dept.*, 1944, **80** (Sept.), 3.

⁶ Havens, W. P., Jr., Ward, R., Drill, V. A., and Paul, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 206.

⁷ Stuart, C. A., *Infectious Mononucleosis (in) Diagnostic Procedures and Reagents*, 2nd Ed., New York, Am. Pub. Health Assn., 1945, p. 449.

TABLE I.

Results of Heterophile Antibody Agglutination Tests in 508 Patients with Viral Hepatitis.

Week of disease	No. of patients	
	Tested	Positive* 1:56
1	11	0
2	116	1
3	167	2
4	192	4
5	158	3
6	153	0
7	132	1
8-12	372	3
13-17	75	1
18-30	37	1

* After absorption on boiled guinea pig kidney, titer was 1:7 or negative. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

TABLE II.

Results of Kahn Tests in 388 Known Non-luetic Patients with Viral Hepatitis.

Week of disease	No. of patients			Week tests became negative
	Tested	Positive*	Doubtful*	
1	70	3	1	2nd, 3rd 14th
2	175	2	4	3rd, 5th, 7th
3	237	0	3	4th, 6th, 7th
4	247	0	1	5th
5	212	1	0	14th
6	195	0	1	7th
7	153	0	0	
8-12	290	0	0	
13-17	58	0	0	
18-30	8	0	0	

* All tests reported as positive or doubtful had less than 10 Kahn units. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

recorded in Table I. The volunteers in Group I, who were all known to have negative tests before experimental inoculation with hepatitis virus, failed to develop positive reactions during the 2nd, 3rd or 4th week after onset of disease. In Group II, none of the patients had titers of heterophile antibody above 1:14 at the 2-hour reading, although 41 patients had titers of 1:28 at the 12-hour reading. Sixteen patients had positive tests at the 12-hour reading with titers of 1:56 which were reduced to 1:7 or negative following absorption on boiled guinea pig kidney.

Kahn tests. Technique of test. The

TABLE III.

Results of Cold Agglutinin Tests in 323 Patients with Viral Hepatitis.

Week of disease	No. of patients		Week test became negative
	Tested	Positive* 1:32	
1	37	0	
2	81	0	
3	106	0	
4	108	0	
5	79	0	
6	79	0	
7	60	1	8th
8	45	1	9th
9-12	111	0	
13-16	40	0	
17-23	16	0	

* Tests reverted to negative after standing 3 hours at room temperature. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

standard and quantitative Kahn tests were carried out according to methods recommended by the U. S. Army.⁸

The results of testing 388 patients in Groups I and II are recorded in Table II. One of the volunteers in Group I developed a positive Kahn test in the 2nd week of disease which became negative in the 7th week. It is of interest to note that this patient had a superimposed bacteremia with *S. cholerae suis*.⁹ In Group II, 358 patients, who were known not to have syphilis, were tested. Six patients developed positive Kahn tests, and 10 had doubtfully positive tests. These tests became negative between the 3rd and 14th week of disease in all patients.

Cold agglutinin tests. Technique of tests. The tests were carried out according to the method recommended by Bray.¹⁰

The results of testing 323 patients in Groups I and II are recorded in Table III.

⁸ Turner, T. B., and Rein, C. R., *Sero-diagnosis of Syphilis*, Chap. XII, (in) Simmons and Gentzkow's *Laboratory Methods of the United States Army*, 5th Ed., Philadelphia, Lea and Febiger, 1944, p. 124.

⁹ Havens, W. P., Jr., and Wenner, H. A., *J. Clin. Invest.*, 1946, **25**, 45.

¹⁰ Bray, W. E., *Synopsis of Clinical Laboratory Methods*, 3rd Ed., 1944, St. Louis, C. V. Mosby Company, p. 163.

tients in the acute phase of hepatitis developed an heterophile antibody which agglutinates sheep erythrocytes in a titer of 1:40 or greater. The titer of this antibody rarely rises above 1:160, and may be differentiated from that appearing in patients with infectious mononucleosis by its absorbability on boiled guinea pig kidney and human liver.

It has also been reported that a certain number of patients with viral hepatitis develop transient falsely positive tests for syphilis,⁴ and as many as 20% of one group of patients were reported to have falsely positive Kahn and Wassermann tests during the course of acute hepatitis.⁵

Such a substantial percentage of positive serologic tests, occurring during the course of hepatitis, might constitute a problem in diagnosis. Certain similarities between hepatitis and infectious mononucleosis, such as the presence of cervical lymphadenopathy and lymphocytosis as well as the frequent occurrence of evidence of hepatic dysfunction in the latter disease, make differential diagnosis more difficult.

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Method and Materials. Subjects. The patients are divided into 2 groups. *Group I:* 30 previously healthy male volunteers, ranging in age from 19 to 29 years, contracted

infectious hepatitis experimentally, following inoculation or ingestion of material known to contain infectious hepatitis virus. The strain of virus used in these experiments has been previously described.⁶ *Group II:* 478 subjects were patients in an Army hospital in Germany from June 1947 to February 1948. They had contracted acute hepatitis during the performance of duty. It is quite probable that many of these cases were examples of homologous serum jaundice, but in view of the fact that there are no means at present for differentiating infectious hepatitis from serum hepatitis clinically, the cases have all been classified as viral hepatitis. The diagnosis of hepatitis was made in both groups on the basis of characteristic symptoms and signs, accompanied by consistent deflection of appropriate tests of hepatic function. All patients in this report had clinical jaundice.

In Group I, the heterophile antibody agglutination, Kahn, and cold agglutinin tests were determined before experimental inoculation. All were negative. These subjects were tested again during the 2nd, 3rd and 4th weeks of disease, and sera with positive reactions were retested at weekly intervals until negative.

In Group II, these tests were performed in various stages of infectious hepatitis, ranging from the 1st to the 30th week. Numerous serial determinations were made, and the majority of tests were done during the first 8 weeks of disease. Sera with positive reactions were retested at weekly intervals until negative.

Results. Heterophile antibody agglutination tests. Technique of test. The tests were carried out according to the method of Stuart.⁷ Sera with a titer of 1:56 were absorbed on boiled guinea pig kidney and retested.

The results of the determinations in 508 patients represented in Groups I and II are

² Eaton, M. D., Murphy, W. D., and Hanford, V. L., *J. Exp. Med.*, 1944, **79**, 539.

³ Olitski, L., and Bernkopf, H., *J. Infect. Dis.*, 1945, **77**, 60.

⁴ Waelsch, J. H., *Brit. Med. J.*, 1946, March 9, 353.

⁵ Kuzell, W. C., and Puccionelli, V., *Bull. U. S. Army Med. Dept.*, 1944, **80** (Sept.), 3.

⁶ Havens, W. P., Jr., Ward, R., Drill, V. A., and Paul, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 206.

⁷ Stuart, C. A., *Infectious Mononucleosis (in) Diagnostic Procedures and Reagents*, 2nd Ed., New York, Am. Pub. Health Assn., 1945, p. 449.

LIGHT AND PIGMENT GROWTH



PLATE I.

All figures shown are photographs of the lower surface of living summer flounders. Each animal is selected as typical for the particular experimental situation.

FIG. 1. Normal control freshly caught. The entire ventral surface is free from melanophores.

FIG. 2. Fish black-adapted 2 days, blinded and maintained in darkness 30 days. Entire surface is white as in the control fish except for a few pigmented regenerated scales.

FIG. 3. Animal black-adapted 3 days, blinded and kept for 41 days in a black tank brightly lighted from above. About 2.0 Weston units of light. Somewhat more pigment has developed here than in either of the above figures.

The volunteers in Group I, who were all known to have negative tests before experimental inoculation with hepatitis virus, failed to develop positive reactions during the 2nd, 3rd or 4th week after onset of disease. In Group II, 2 patients had positive tests with a titer of 1:32, which reverted to negative after standing at room temperature for 3 hours.

Summary. Heterophile antibody agglutination, Kahn, and cold agglutinin tests were performed in 2 groups of patients with viral hepatitis. Sixteen out of 508 patients (3%) developed positive heterophile antibody tests with titers of 1:56 which were

reduced to 1:7 or negative by absorption on boiled guinea pig kidney. A rise and/or fall in titer of antibody was demonstrable in serial weekly determinations. Out of 388 patients who were known not to have syphilis, 6 (1.5%) developed positive Kahn tests, and 10 others (2.5%) had doubtful tests. Two out of 323 patients had cold agglutinins present in a titer of 1:32.

The relatively small number of positive heterophile antibody agglutination and Kahn tests is in contrast to reports of others^{2,5} who have found a considerably higher percentage of positive tests.

16334

Factors Influencing the Growth of Integumentary Pigment in Fishes. I. The Role of Light.*†

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It is a common observation that light is associated with changes in body pigmentation such as "tanning." Numerous clinical observations¹⁻⁵ indicate that certain types of pigmentation deficiencies in man may be corrected by light treatment. In lower mammals Bissonnette and others have demonstrated that pigmentation may be influenced by il-

lumination while experimental evidence has accumulated indicating the importance of light in producing integumentary pigmentation in lower vertebrates. Cunningham⁶⁻⁸ working with flatfishes; Herbst and Ascher⁹ using salamander larvae; Vilter¹⁰ studying axolotls; Sumner and Wells;¹¹ Sumner and Doudoroff;¹² Odiorne;¹³ Sumner;¹⁴⁻¹⁶ Os-

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† Contribution No. 427 of the Woods Hole Oceanographic Institution whose research facilities were generously provided for this investigation.

‡ Now located at Syracuse University.

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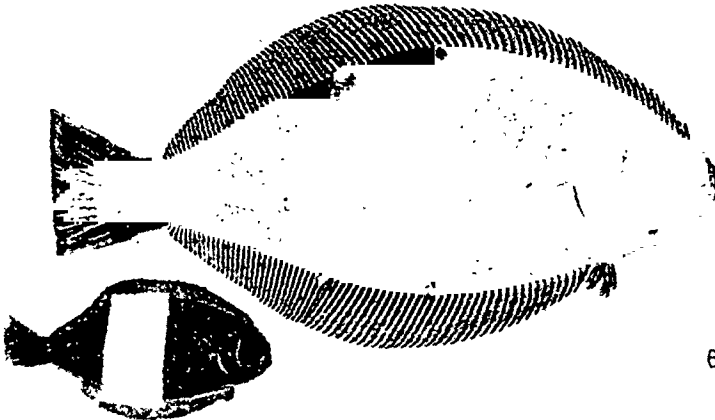
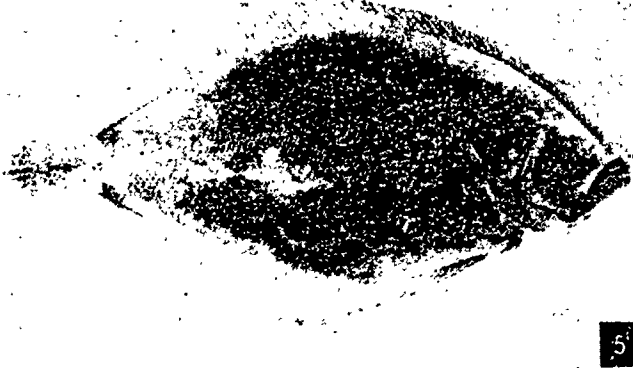


PLATE II.

All photographs are of the normally unpigmented ventral surface of living summer flounders. All inserts are further reductions of the corresponding animal.

FIG. 4. Summer flounder black-adapted $2\frac{1}{2}$ days, blinded and placed in a brightly illuminated white tank (reflecting about 16 Weston units) for 28 days. A considerable increase in ventral melanophore production over that seen in the previous figures.

FIG. 5. Black-adapted 2 days, blinded and maintained in the special apparatus providing direct ventral illumination of high intensity (about 400 Weston units) for 26 days. First evidence of melanin formation was noted at about 90 hours. Note that the ventral surface has become quite completely darkened by an extensive covering of melanophores.

FIG. 6. This fish was maintained under the same conditions as described for Fig. 5. In addition an opaque mask was affixed to cover part of the ventral surface (see insert). This mask was kept in place during the entire sojourn of the animal in the ventral illumination apparatus (26 days). The mask was removed and the photograph taken at the end of that time. The area protected from the light is free from melanophores.

born;¹⁷⁻²¹ and Dawes²² experimenting with various species of fishes have all reported experiments concerning integumentary pigmentation in which light was an important factor. In recent work¹⁷⁻²¹ it was found that melanophore development in normally non-melaninated areas depended upon the maintenance of two general conditions. First, the internal environment must be one which favors darkening in the normally pigmented areas; and, second, the external environment must provide illumination (direct or by reflection) to the integument.

The experiments reported here were designed to obtain more detailed information on the role of light in the development of melanophores on the normally unpigmented surface of the summer flounder.

Materials and Methods. Summer flounders freshly caught from Woods Hole waters were used for the experiments. The water temperature for all experiments ranged between 18 and 21°C. The experimental fishes were background-adapted in black tanks illuminated artificially from above. Light intensity (reflected or transmitted) was measured by a Weston exposure meter. The following modifications were made on the ventral illumination tubs previously described:¹⁷ (a) removable false bottoms of perforated glass were rested upon glass supports $\frac{3}{4}$ inches above the permanent glass floor to allow water to circulate between the 2 glass plates and protect the lower surface of the fishes from overheating; (b) three independent water inlets near the bottom of the tub provided rapid circulation and adequate overflows connected to variable level standpipes allowed easy adjustment of the water level; (c) the light intensity was increased by using a total of

600 Watts for each glass bottomed tub and the walls and ceiling of these special tubs were white to produce a high incidence of internal reflection.

After being black-adapted, the experimental flounders, 106 in all, were totally blinded by enucleation. Opaque masks of black cloth were held in position by suturing each corner to the base of an adjacent fin ray. This kept the mask securely in place without hampering the normal movements of the fishes. Photographic records of the living fishes were made at frequent intervals, supplemented by others of preserved animals.

Adequate unoperated control animals (Fig. 1) in a natural environment were available for purposes of comparison as the experiments progressed.

Experimental. Summer flounders previously black-adapted and totally blinded were placed in total darkness (Exp. 1); subjected to different amounts of overhead illumination where mostly reflected light reached the ventral surface (Exp. 2, 3 and 4); exposed to direct intense illumination ventrally without masking local areas (Exp. 5) or protecting parts of the ventral surface with opaque masks (Exp. 6).

Exp. 1. Summer flounders black-adapted then blinded as previously described were kept in total darkness from 2 to 6 weeks. Without exception these animals failed to develop any general melanination on the ventral surface (Fig. 2). Here and there a black scale did appear but all were cases of regenerating scales where the original had been lost during experimental manipulation. This will be considered as a constant in subsequent experiments described here. The pigmentation of regenerated scales comprises a special situation which must not confuse the objectives of these experiments.

Exp. 2, 3, 4. These 3 experiments embraced changes in intensity of light source and shade of background so that the light reflected from the surfaces was 0.1 to 0.2, 2.0, and 16.0 Weston Units respectively. Considering the findings from these experiments collectively it was observed that the most ventral melanin appeared on those fishes illuminated most intensely and long-

¹⁵ Sumner, F. B., *J. Exp. Zool.*, 1940a, **83**, 327.

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¹⁷ Osborn, C. M., *Proc. Nat. Acad. Sci.*, 1940a, **26**, 155.

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²⁰ Osborn, C. M., *Biol. Bull.*, 1941a, **81**, 341.

²¹ Osborn, C. M., *Biol. Bull.*, 1941b, **81**, 352.

²² Dawes, B., *J. Exp. Biol.*, 1941, **18**, 26.

melanophores of intact scales.

2. Light passing directly through glass bottomed tanks onto the lower surface of the flounders was the most effective in producing melanophores. This means of illumination delivered the most intense light to the ventral surface of the fish and could be most accurately measured.

3. The use of opaque masks indicates that light affects the cell directly to produce melanin.

4. The melanination of regenerating scales is apparently not dependent upon the presence of light. Investigation of this special problem is being continued.

16335

Technique for the Biomicroscopic Study of the Ovary and the Fallopian Tube.

CLEMENTE ESTABLE.

From the Instituto de Investigación de Ciencias Biológicas, Montevideo.

In order to explore the ovary microscopically *in vivo* a method is described, the first step of which is the eventration of the ovary, with or without the Fallopian tube, and its placing in a subcutaneous loggia. To do this, the animal (rabbit) is fastened on its back, with the posterior part of its body rotated 30° to 45° on the longitudinal axis. Ether or some other anesthetic is administered. Then the ovary, with or without the Fallopian tube, is reached via a discreet section of the abdominal wall, slightly under the dorso-lateral line projected by the course of the Fallopian tube, and behind the kidney. Then the great external oblique muscle is pierced by means of a surgical knife or the tip of a scissors slightly above this first incision, and the broad ligament is gently clamped; without being touched, the ovary is thus eventrated through the small opening in the muscle, the aponeurosis of which serves as a bed. All suturing close to the ovary should be avoided and is not necessary, as the ovary remains in its new position by simply placing it perpendicularly to the incision (Fig. 1). When removing the ovary from the abdomen great care must be taken not to provoke any circulatory or nervous disturbances; this point is of the utmost importance as regards all further activity of the ovary. The wound in the skin should not

coincide with the ovarian surface or with the wound in the muscle which forms the bottom of the subcutaneous loggia. A flap-like portion of skin should cover the ovary, or the Fallopian tube, or both, as the case may be. Neither the suture of the muscle nor that of the skin should be in contact with the organs in the subcutaneous loggia. Thus, the ovary, the tube, or both can be easily reached without causing trauma. The best material for study is that in which no adhesion between skin and ovary arises; this is usually prevented by the secretions of the transferred organs, as well as by the frequent opening of the loggia necessary for repeated macro and microscopic studies of the ovary and the Fallopian tube.

Visualization capsule. The first experiment performed tried to keep the ovary visible through a grafted cornea. This procedure was a failure.

The visualization capsule is made up of 4 parts: (a) an ebonite ring of varying diameter and height, threaded on the inside and having a flat perforated base which allows it to be sutured under the skin, and large enough to accommodate the ovary and the Fallopian tube; (b) a second ring which screws into the first to which is fastened an extremely fine, transparent and elastic, removable diaphragm; 2 small holes in the ring

est. Fig. 3 and 4 illustrate typical cases.

Exp. 5. In this experiment intense light (400 Weston Units or 25X as much as was used in Exp. 4) was directed to the ventral surfaces of the fishes through glass bottomed tanks. This method provides two significant advantages. First, it is possible by using various lamps to subject the lower surface of the experimental animal to any intensity desired; and second, it is possible to measure with accuracy the amount of light falling directly upon the lower surface of the fish. Thus, illumination may be rigidly controlled.

Under such favorable conditions pigment formation proceeded rapidly. Frequently it was possible to detect some evidence of melanophore formation during the fourth day of illumination and after 4 weeks of such treatment the entire ventral surface was found to be generally darkened with an extensive growth of melanophores (Fig. 5).

Exp. 6. Success in blackening the ventral surface so completely with melanophores in a relatively short time suggested an experiment designed to determine whether the pigmentation response was due to the *direct* effect of illumination, assuming the internal environment to be optimum in all the experiments, or whether it was necessary only that light be present for the reaction to continue. It was reasoned, therefore, that if it were necessary for light to fall directly upon the cell (direct effect) areas covered by an opaque mask would remain unpigmented while exposed areas would develop melanophores as in Exp. 5. On the other hand if it was necessary only that a fish with optimum internal physiological conditions be generally illuminated one might expect that the pigmentation reaction would go on underneath the mask regardless.

Accordingly, flounders prepared as in Exp. 5 were further provided with opaque masks as described previously (Fig. 6—insert). They were illuminated ventrally (400 Weston Units) as before and simple, clean-cut results were obtained. The exposed surfaces became melaninated but the protected area remained as free from pigmentation as in the control fishes. This striking result seen in Fig. 6 seems to indicate clearly that light

provides a direct stimulus to the particular cell destined to develop into a melanophore by producing melanin.

Discussion. It becomes clear from the data presented that light is not only a very significant factor in the experimental development of melanophores in a normally unpigmented area but also that there is a correlation between the amount of pigment grown, the intensity of the light and duration of the exposure. Data of a sufficiently quantitative nature to determine whether this relationship is direct over all ranges of light intensity are not available. It is probable, however, that a direct relationship between light intensity per unit time and degree of melanination would not hold in ranges of very high intensity. The physiological limit of the rate at which melanin could be formed would probably be reached before arriving at extremely high intensities of light.

The impression should not be gained from these experiments, however, that light is the most important single factor in the production of melanophores. Although it is obviously an essential factor (except perhaps in the case of regenerated scales) it must be emphasized that the condition inside the cell as evidenced by the degree of dispersion of melanin granules in the cells of normally pigmented areas must represent the darkening phase of the fish. The ineffectiveness of high intensities of light directed on the surface of a flounder while in a physiological condition not favoring darkening is strikingly shown in the case of a white-adapted fish where, instead of melanin production one witnesses an actual decrease in dark pigmentation due to melanophore degeneration.^{12,13,22} The observation that in physiologically dark flounders regenerating scales develop melanophores even in the absence of illumination is further evidence that in certain instances light is not necessary for the growth of melanin in melanophores. The problem of the pigmentation of regenerating scales represents a special case which deserves more detailed investigation in the future.

Summary. 1. In addition to a favorable internal environment in the cell, light is necessary for the development of melanin in

phragm affords poor protection of the ovary against drying.

The subcutaneous ovary may be examined daily with the microscope,—the binocular, the ultrapaque or a mono-objective type; in the latter case a microlamp, an illuminating needle or a thin spatule of plastic material is placed directly beneath it or by perforating the tissues under the ovary.

When, through an error in operating, the hind leg of the rabbit rubs against the capsule, the sciatic nerve is sectioned to avoid irritation of the ovary, thus paralyzing the leg.

Precautions must be taken against vascular block,—as this might cause gangrene,—and against inflammatory irritations or damage to the nervous fibers. Obviously, this is not a subcutaneous graft of the ovary; it is a careful and adequate eventration, which maintains circulation and innervation intact.

With the technique for the biomicroscopic examination of the ovary and the Fallopian tube presented above, it is possible to follow, under the microscope, the development of the Graafian follicle, its circulatory changes, the exact moment when the "stigma" appears, its dehiscence, the flow of the follicular liquid carrying away with it the oöcyte, and the peristalsis of the Fallopian tube.

Observations already made are being prepared for publication.

Summary. For the microscopic observation *in vivo* of the mammal ovary and the Fallopian tube, two techniques are described, with special reference to the rabbit: (1) the transference of the ovary to a subcutaneous loggia, (2) the eventration of the ovary and its protection by a visualization capsule through which microscopic examination is possible. In both cases nervous and vascular connections are kept intact.

16336 P

Effect of Sodium Bicarbonate and Ammonium Chloride on Ascorbic Acid Metabolism of Adults.

BETTY E. HAWTHORNE* AND CLARA A. STORVICK.* (Introduced by J. S. Butts.)

From the School of Home Economics, University of Washington, Seattle, Washington.

A consistent lowering of the urinary ascorbic acid excretion of adults has been observed when an increased urinary pH is effected by the ingestion of sodium bicarbonate.^{1,2,3} Guinea pig experiments measuring "scurvy scores" and body stores of ascorbic acid⁴ indicated that the lowered excretion of ascorbic acid as a result of the ingestion of sodium bicarbonate appeared to represent an increase

in retention rather than greater destruction as might be supposed from *in vitro* experiments. This communication reports for the first time, as far as we know, the results of simultaneous daily determinations of plasma and urinary ascorbic acid concentrations of adults on controlled ascorbic acid intakes ingesting sodium bicarbonate or ammonium chloride.

* Present address, School of Home Economics, Oregon State College, Corvallis, Oregon.

¹ Heinemann, M., *J. Clin. Invest.*, 1941, **10**, 39.

² Hawley, E. E., Stephens, D. G., and Anderson, G., *J. Nutrition*, 1936, **11**, 135.

³ Patterson, I., unpublished data, Cornell University, Ithaca, N.Y., 1942.

⁴ Hawley, E. E., Daggs, R. G., and Stephens, D. J., *J. Nutrition*, 1937, **14**, 1.

Experimental. The subjects were 2 adult women, C. C. and J. G., in normal health, both actively interested in nutrition research. The experiment was divided into 3 periods of 14 days each: 1st period—basal diet⁵ (containing by analysis 7 mg of ascorbic

⁵ Lewis, J. S., Storvick, C. A., and Hauck, H. M., *J. Nutrition*, 1943, **25**, 185.



FIG. 1.
Ovary (A) transferred to the great oblique muscle of the abdomen (B). The eventration was performed through a small opening. The ovary stays *in situ* without ligature or suture.



FIG. 2.
Eventrated ovary, within the fixed part of the visualization capsule.



FIG. 3.
Ovary lying inside the visualization capsule, protected by a transparent, elastic diaphragm.

serve to screw or unscrew it; (c) an ebonite lid which screws onto the outside of the upper portion of the first ring, closing the ovarian chamber hermetically (Fig. 2, 3); it can be removed very easily every time it is necessary to make macro and microscopic observations of the ovary; (d) a metal key with 2 fine spigots which fit into the holes in parts (b) and (c) for screwing or unscrewing. This mobility and the elasticity of the diaphragm undoubtedly facilitate biomicroscopy

and make preservation of the ovary very easy. The rigid diaphragm prevents the frontal lens of the objective from being brought as close to the ovarian surface as one would wish, becoming blurred more rapidly and being more difficult to replace. The construction allows the removal of parts and whenever necessary the examination of the ovary without any diaphragm. The lid protects the diaphragm and insures better asepsis; the capsule with the lid and without the dia-

ammonium chloride increased the urinary excretion of ascorbic acid. The mean daily urinary ascorbic acid excretions for the 3 periods are: C. C. 1st period, 26 ± 1.3 mg, 2nd period, 19 ± 2.0 mg, and 3rd period, 44 ± 4.1 mg; and J. G. 1st period, 41 ± 2.7 mg, 2nd period, 34 ± 7.6 mg, and 3rd period, 46 ± 4.3 mg. The differences between the periods were significant for C. C. but not J. G. tested according to Livermore's formula: differences must be 2 times the standard deviation of the differences.⁸ The mean daily plasma ascorbic acid levels were consistently and significantly⁸ decreased from the levels of the regular control diet with the addition of sodium bicarbonate or ammonium chloride. The mean daily plasma ascorbic acid values for the 3 periods are: C. C. 1st period, $0.98 \pm .03$ mg %, 2nd period, $0.87 \pm .02$ mg %, 3rd period, $0.89 \pm .02$ mg %; and J. G. 1st period, $0.98 \pm .02$ mg %, 2nd period, $0.81 \pm .04$ mg %, and 3rd period, $0.79 \pm .01$ mg %.

Hathaway and Meyer⁹ have defined "utilization" as the difference between intake and

excretion. By this definition the subjects' apparent "utilization" on the 3 periods was as follows: C. C., 61%, 72%, and 34%, and J. G., 39%, 49%, and 31%, respectively. Apparent "utilization" is then increased with the administration of sodium bicarbonate and decreased with ammonium chloride ingestion. However, the lowered mean plasma ascorbic acid values and the varied plasma and urinary excretion responses to the administration of a test dose at the completion of each period (Fig. 1) are not consistent with the apparent percentage "utilization."

Summary. This study indicates that the changes in the excretion of ascorbic acid are not accurate indications of utilization. The ingestion of both salts significantly lowered the mean plasma ascorbic acid content of the subjects indicating an interference with normal utilization. The reduced excretion of ascorbic acid with the ingestion of sodium bicarbonate both daily and in response to a test dose would seem therefore to represent more accurately increased destruction of ascorbic acid in the excretory process; and conversely, the increased excretion with ammonium chloride would seem to represent increased preservation in the excretory process.

⁷ Farmer, C. J., and Abt, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 1625.

⁸ Hauck, H. M., personal communication, 1943.

⁹ Hathaway, M. L., and Meyer, F., *J. Nutrition*, 1941, **21**, 503.

16337

Application of a Metabolic Inhibitor to the Developing Chick Embryo.

W. WILBUR ACKERMANN* AND ALFRED TAYLOR.

From the Biochemical Institute, The University of Texas, and the Clayton Foundation for Research, Austin.

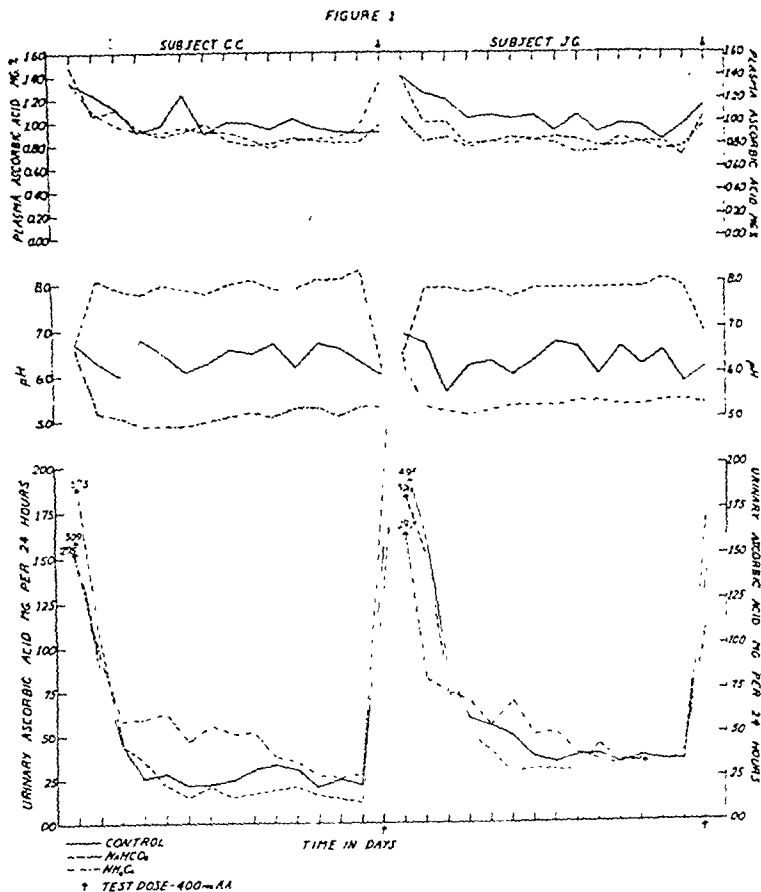
This research is the first of a series of attempts to apply a new method to the study of chemical embryology. The usefulness of metabolic analogs (anti-vitamins, anti-metabolites) in the study of the metabolism of microorganisms has been fully demonstrated by Shive and his co-workers in this laboratory

by the development of the method of "inhibition analysis".^{1,2} An outgrowth of these findings, which has been in prospect in this laboratory for some time, is an attempt to block metabolic reactions in embryonated and cancer-bearing eggs and to apply the same methods of study to these tissues. A study of the effect of precursors of the competing metab-

* Rosalie B. Hite Fellow.

¹ Shive, W., and Macow, J., *J. Biol. Chem.*, 1946, **162**, 451.

² Beerstecher, E., and Shive, W., *J. Biol. Chem.*, 1946, **164**, 53.



THE EFFECT OF SUPPLEMENTS OF AMMONIUM CHLORIDE AND SODIUM BICARBONATE ON THE URINARY pH, URINARY EXCRETION OF ASCORBIC ACID AND PLASMA ASCORBIC ACID CONTENT OF TWO HUMAN SUBJECTS

FIG. 1.

acid) plus 60 mg of crystalline ascorbic acid daily; 2nd period—basal diet plus 60 mg of ascorbic acid plus 15 g NaHCO₃ daily; and 3rd period—basal diet plus 60 mg of ascorbic acid plus 4 g NH₄Cl daily. Preceding each experimental period the subjects were saturated with ascorbic acid⁶ and each period was concluded with a determination of response to a 400 mg test dose. The 60 mg supplement of ascorbic acid was dissolved in water and taken just before breakfast; the 15 g of sodium bicarbonate were given as 5 g portions dissolved in water at 10 a.m., 3 p.m. and 8

p.m.; and the 4 g of ammonium chloride were given as 2 g portions dissolved in water at 10 a.m. and 3 p.m. The pH and ascorbic acid determinations were made on 24-hour urine collections according to the method described by Belser, Hauck and Storvick.^{6†} Fasting plasma ascorbic acid values were determined by the method of Farmer and Abt.⁷

Results. The results are illustrated in Fig. 1. When compared with the control period, sodium bicarbonate decreased and

⁶ Belser, W. B., Hauck, H. M., and Storvick, C. A., *J. Nutrition*, 1939, **17**, 513.

[†] The authors wish to acknowledge the assistance of Catherine Cobb and Josephine Graham for these determinations.

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⁷ Farmer, C. J., and Abt, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 1625.

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⁹ Hathaway, M. L., and Meyer, F., *J. Nutrition*, 1941, **21**, 503.

16337

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² Beerstecher, E., and Shive, W., *J. Biol. Chem.*, 1946, **164**, 53.

olite on such an inhibitor in embryonic tissue holds promise of information as to the embryo's biochemical abilities, as well as affording a means of following the development of the biochemical potentialities of the embryo in much the same manner that the morphological development has been traced.

The inhibitor, 3-acetylpyridine, used in this investigation has already found use in the production of pellagra-like symptoms in the mouse, an animal normally requiring no exogenous source of nicotinic acid.³ It has been reported that its toxicity on this animal can be nullified by feeding nicotinic acid or tryptophan which have been postulated as precursors of nicotinamide.^{4,5}

Developing chick embryos are inhibited by 3-acetylpyridine and this inhibition can be reversed competitively by nicotinamide, but nicotinic acid and tryptophan are much less active reversing agents. Details of some of these experiments with embryonic chicks and their implications are presented below.

Testing Methods. All solutions, injected into eggs were adjusted to a pH of 7 and were equivalent in osmotic pressure to .58% saline. Sterilization was accomplished by autoclaving and the same precautions of aseptic technique were used in yolk-sac injection as are described for tumor in egg cultivation.⁶ Deaths were determined by candling. Time and temperature of incubation are given in the tables.

Results. The results shown in Table I indicate that 600 γ of 3-acetylpyridine per egg is lethal in 24 hours when injected into the yolk-sac. Sub-lethal doses of 3-acetylpyridine or lethal doses rendered just sub-lethal by the addition of nicotinamide or precursors of this vitamin, cause a mal-development of the chick. Some of the symptoms associated with the toxicity were undersized deformed legs, and a general edematous-like condition over the surface of the body. These symptoms could be prevented by the simultaneous

injection of large amounts of nicotinamide with the 3-acetylpyridine.

Reversal of the Lethal Effect. The amount of nicotinamide necessary to just nullify the lethal effect of 3-acetylpyridine increases as the concentration of this inhibitor increases. Further, from Table I it can be seen that the relationship of these 2 substances is a competitive one and that the inhibitor-substrate (3-acetylpyridine-nicotinamide) ratio is approximately 14. In order to determine this ratio, it is necessary to measure the total inhibitor and substrate which is just non-lethal to the egg. However the egg itself contains some nicotinamide and for this a correction must be made by deducting from the total 3-acetylpyridine that amount which corresponds to the maximum non-lethal dose.

The justification for determining the inhibition constant in this manner can be demonstrated concisely if we allow:

I = Total inhibitor

S = Total substrate

S_a = Added substrate

S_o = Substrate present in egg initially

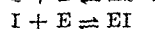
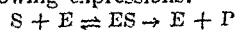
I_o = Maximum non-lethal level of inhibitor

E = Enzyme

K = Inhibitor-substrate ratio

P = Product of substrate

The competition of inhibitor and substrate for some specific enzyme has been represented by the following expressions:



For such a competitive system by definition, the ratio of I to S is a constant

$$(1) \quad \frac{I}{S} = K$$

Since S represents the total amount of nicotinamide present, and this is composed of the vitamin already in the yolk plus that which has been injected then:

$$(2) \quad S = S_a + S_o$$

From expression (1) and (2) one obtains:

$$\frac{I}{S_a + S_o} = K$$

or by rearrangement:

$$(3) \quad I = KS_a + KS_o$$

Now from equation (3) it is obvious, that when the added substrate is zero, the amount of inhibitor which the eggs will tolerate is

³ Woolley, D. W., *J. Biol. Chem.*, 1945, 157, 455.

⁴ Woolley, D. W., *J. Biol. Chem.*, 1946, 162, 179.

⁵ Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, 163, 343.

⁶ Taylor, A., Thacker, J., and Pennington, D., *Science*, 1942, 96, 342.

TABLE I.
Effect of 3-Acetylpyridine and Nicotinamide on 4-day-old Chicks.

Nicotinamide γ per egg	3-Acetylpyridine γ per egg	No. of eggs* injected	Exper. fraction dead in 24 hr	Control† fraction dead in 24 hr	$\frac{I - I_0}{S_a}$
—	400	8	0.00	0.00	—
—	450	8	0.13	0.00	—
—	500	7	0.14	0.00	—
—	550	7	0.14	0.14	—
—	600	8	1.00	0.00	—
80	2000	7	0.72	0.00	—
90	2000	7	0.43	0.00	—
100	2000	7	0.00	0.00	14.5
160	3000	7	0.72	0.00	—
180	3000	7	0.00	0.00	13.6
200	3000	7	0.00	0.00	—
200	4000	8	1.00	0.00	—
220	4000	8	0.63	0.00	—
230	4000	7	0.57	0.14	—
240	4000	8	0.00	0.00	14.4
280	5000	8	0.50	0.00	—
313	5000	8	0.00	0.00	14.2
325	5000	8	0.00	0.00	—
350	6000	8	1.00	0.00	—
370	6000	8	0.75	0.00	—
380	6000	8	0.00	0.00	14.3

* 4-day-old eggs were used, incubated at 37°C.

† Control eggs were injected with .58% sterile saline.

‡ These values were calculated; I and S correspond to the inhibitor and substrate added and I_0 is the maximum non-lethal dose experimentally determined as 550 γ per egg.

K S_0 and represents the largest non-lethal dose. Designating S_0K as I_0 and substituting in expression (3) one has by rearrangement:

$$(4) \quad \frac{I - I_0}{S_a} = K$$

In this last equation the inhibitor-substrate ratio, K, is expressed in all experimentally measurable quantities.

From the experimental value of I_0 and the average experimental value of K, the quantity of S_0 can be estimated, since by designation in expression¹

$$I_0 = S_0K$$

The value of S_0 so estimated for eggs averaging 56 g was found to be approximately 39 γ. The value of Cheldelin and Williams⁷ determined by microbiological assays is .72 γ per gram or 40.3 γ per 56 g eggs.

⁷ Cheldelin, V. H., and Williams, R. J., *The University of Texas Publication*, 1942, No. 4237, 105.

Effect of Nicotinic Acid and Tryptophan.

The effects of nicotinic acid and tryptophan on the toxicity of 3-acetylpyridine on 4-day-old embryos is shown in Table II. It is striking that these two substances while active against low levels of inhibitor are much less potent than the nicotinamide. This does however demonstrate that the 4-day-old chick has the mechanism for the biochemical conversion of nicotinic acid and tryptophan to nicotinamide and that the ability is very feeble. This might indicate that at this stage of development the concentration of the enzymes necessary for the conversion of nicotinic acid to its amide is very low.

Summary. 1. Embryonic chicks were inhibited by the direct injection of 3-acetylpyridine into the yolk-sac. 2. It was shown that this inhibition could be reversed competitively by nicotinamide over the range of 2000 to 7000 γ per egg. 3. Evidence is presented showing that 4-day-old chick embryos have

TABLE II.
Effect of Nicotinic Acid and Tryptophan on the Toxicity of 3-Acetylpyridine.

3-Acetylpyridine γ per egg	Nicotinic acid γ per egg	Tryptophan γ per egg	No. of eggs* injected	Fraction dead in 24 hr
1500	0	0	6	1.00
1500	2000	0	6	0.50
1500	2500	0	6	0.67
2000	0	0	7	1.00
2000	2000	0	7	1.00
2000	2500	0	7	0.88
600	0	0	7	0.86
600	0	7500	7	0.00
650	0	0	7	1.00
650	0	7500	7	0.00
700	0	0	7	1.00
700	0	7500	7	0.14

* All eggs incubated at 37°C and were 4 days old at time of injection.

a very limited capacity to convert nicotinic acid to the corresponding amide. 4. Sublethal levels of 3-acetylpyridine are shown to cause a mal-development of the chick embryo.

16338

Effect of pH on MM Virus.

ALBERT SCHATZ* AND HILDEGARD PLAGER. (Introduced by Grace M. Sickles.)

From the Division of Laboratories and Research, New York State Department of Health, Albany.

MM virus was originally isolated from a hamster which had been injected intracerebrally with cord and medulla of a fatal case of poliomyelitis. Typical and severe poliomyelitic lesions are induced by it in the central nervous system of hamsters and albino mice.¹

Serologically MM virus appears to be similar to the SK virus of Jungeblut and Sanders² but differs from the Lansing-like poliomyelitic strains and the Theiler virus.³

* Present address: The Sloan-Kettering Institute for Cancer Research, Memorial Hospital, New York City.

¹ Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

² Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

³ Dalldorf, G., to be published.

The virus is highly virulent for mice by the intraperitoneal as well as the intracerebral route. Thus it affords an opportunity for testing the effects of antibiotic agents on small amounts of a virus which induces poliomyelitic lesions in the test animal. In relation to a problem of this kind⁴ an investigation was made of the effect of pH on MM virus.

Procedure. Mouse brains from the 45th to the 48th generation stored in 50% glycerol were ground, made up in a 10% suspension usually in salt solution containing 10% of infusion broth or occasionally in distilled water, and centrifuged to remove large particles. The supernatant fluid was used at once or after

⁴ Schatz, A., and Plager, H., *Bull. Torrey Bot. Club*, 1948, **75**, 256.

storage in the dry-ice box. Albany standard strain mice[†] weighing 10 to 12 g were injected intraperitoneally. By this route of inoculation the majority of animals given 0.05 ml of a 0.5×10^{-6} dilution of mouse brain were usually dead or paralyzed by the seventh day. Unless otherwise specified, animals were observed over a period of one week after which experiments were discontinued. Five mice were employed for each individual test.

The buffers were Michaelis' veronal acetate,⁵ Sorenson's glycocoll sodium hydroxide,⁶ and the phthalate, phosphate, borate, and hydrochloric acid mixtures of Clark and Lubs.⁶ These were made up approximately to the desired pH and sterilized by heating for 10 to 15 minutes in a water bath or by autoclaving for 15 minutes at 15 pounds' pressure. In most instances, 0.5 ml of a 10^{-3} distilled-water dilution of virus-infected mouse brain was added to 4.5 ml of buffer. The effect of distilled water and of physiologic saline adjusted to different reactions with acetic or hydrochloric acid was also determined. After the desired periods of incubation at room temperature, dilutions were made in broth-salt solution and tested in mice.

Final pH values for the suspensions were determined electrometrically on corresponding normal mouse-brain mixtures, except for the first test with veronal acetate and glycocoll solutions in which the effect of buffer dilution was duplicated with distilled water instead of normal mouse-brain suspension. Bromphenol and bromthymol blue indicator tests with 10^{-4} dilution of virus mouse brain and normal mouse brain in veronal acetate buffer at pH 4.0 and 7.0 and also in aqueous suspensions at pH 7.4 revealed no difference in reaction between the normal and infected mouse-brain suspensions. For solutions at pH 9.0 and above, corrections for sodium-ion

TABLE I. Survival of MM Virus in Veronal-acetate and Glycocoll NaOH Buffers. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	Exp. No.	pH	Period of exposure, days									
			1		2							
			Virus dilutions inoculated into mice		Virus dilutions inoculated into mice							
			10-4	10-5	10-6	10-6	10-5	10-6	10-4	10-5	10-6	10-6
Veronal-acetate buffer	2	2.02	0/5	0/5	0/5							
"	"	3.48										
"	"	4.05	2/5*	0/5	0/5				0/5	0/5		
"	"	4.15		0/5	1/5		2/5	0/5	0/5	0/5		
"	"	5.17		0/5	0/5		0/5	0/5	0/5	0/5		
"	"	6.18										
"	1	7.15		5/5	5/5		5/5	5/5	5/5	5/5	4/5	
"	1	8.04		5/5	5/5		5/5	5/5	5/5	5/5	5/5	
"	1	8.95		5/5	4/5		5/5	5/5	5/5	5/5	5/5	
Glycocoll-NaOH buffer	1	8.99		5/5	5/5		4/5	3/5	5/5	5/5	5/5	
"	1	9.95		5/5	5/5		5/5	4/5	3/5	1/5	0/5	
Control (distilled water)	1			5/5†	5/5		5/5	5/5	5/5	5/5	5/5	
"	2			5/5	3/5					5/5	3/5	
"		7.40										

Numerator = number of mice which died; denominator = number of mice injected. Test period = 7 days.

* 2 survivors paralyzed on 7th day. † 2 paralyzed mice sacrificed on the 3rd day. ‡ 1 paralyzed mouse sacrificed on the 3rd day.

[†] A strain used at the Division of Laboratories and Research, New York State Department of Health, Albany, New York.

⁵ Michaelis, L., *Biochem. Z.*, 1931, **234**, 139.

⁶ Clark, W. M., *The Determination of Hydrogen Ions*, Baltimore, Williams and Wilkins, 1928, 717p.

TABLE II. Survival of MM Virus in HCl, Phthalate, Phosphate, and Borate Buffers. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	Exp. No.	pH	Period of exposure, days						
			1		2				
			10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
HCl	3	1.12	0/5	0/5	0/5				
Phthalate buffer	3	2.10	1/5	0/5	0/5				
	3	3.00	0/5	0/5	0/5				
	3	4.00	1/5	0/5	0/5				
	3	5.00	0/5	0/5	0/5				
Phosphate buffer	4	6.00		3/5	1/5	2/5	3/5	0/5	5/5
	4	7.01		5/5	5/5	5/5	5/5	5/5	5/5
	4	7.75		5/5	5/5	4/5	5/5	4/5	0/5
	4	7.71		4/5*	5/5	4/5	5/5	5/5	4/5
Borate buffer	4	8.75		5/5	4/5	5/5	5/5	5/5	5/5
	4	9.55		5/5	4/5	3/5	3/5	0/5	0/5
	3	7.01		4/5	3/5			5/5	2/5
	4	7.47		5/5	5/5	5/5	3/5*	5/5	3/5
Control (distilled water)									

Numerator = number of mice which died; denominator = number of mice inoculated. Test period = 7 days.
 * 1 survivor paralyzed. ± 0.05 .

concentration were taken into account. Inoculation of mice with virus-free buffers at the extremes of pH indicated no toxicity for the solutions *per se*. When streaked on blood-agar plates, the mixtures gave no growth.

Experimental Results. Tables I and II indicate the pH tolerance of MM virus in the different buffers at room temperature. These results and other data not presented here show that under the conditions of the experiment the virus when exposed at 10^{-4} dilution was stable in the neutral and alkaline solutions. There was a definite loss of activity within twenty-four hours in the veronal-acetate and phosphate buffers below pH 7. On the other hand, the virus survived for at least 2 days in glycocoll and borate solutions at pH 9.95 and 9.55, respectively, but a decrease in activity was evident when tested on the 7th day. The veronal-acetate buffer, at approximately pH 7.8, contained infective virus in the 10^{-6} dilution after seven days at room temperature followed by refrigeration for 8 days. There was no significant difference between the phosphate and phthalate buffers at pH 6.0. However, the results did indicate that virus survival was somewhat better at around pH 8.0 in the borate than in the phosphate medium; at 10^{-4} dilution, active virus was present in the former but not in the latter buffer after 7 days at room temperature followed by 10 days at 4° - 6° C.

In a more detailed experiment, ten-fold virus dilutions from 10^{-2} through 10^{-6} or 10^{-7} in broth salt solution, were exposed to equal amounts of veronal-acetate buffers. Similar dilutions prepared with normal mouse brain had pH values of 4.35, 5.25, 6.35-6.45, 6.85-6.95, 7.75-7.8. After incubation for 1- and 7-day periods, suitable ten-fold dilutions were inoculated into mice. The results indicated, in general, a less deleterious effect of unfavorable pH in the more concentrated virus suspensions. The virus stability was definitely greater at pH 7.8 than at pH 6.9. In this experiment and in several others, there was occasional evidence of some virus survival at around pH 4.0 as compared to that at pH 5.0. While these results are suggestive of greater virus stability somewhere

in the vicinity of pH 4.0 than at acid reactions immediately above or below, more detailed studies would be required to ascertain whether this is actually so.

Whether the increased virus survival in the lower dilutions and the apparent occasional survival at approximately pH 4.0 were due to a protective action of the mouse-brain constituents is not known. No visible flocculation of the mouse-brain suspensions occurred in the buffers with 10^{-4} dilution. However, at a dilution of 10^{-2} , maximum precipitation was observed at pH 3 to 5. This is in agreement with a similar observation of Wenner⁷ on flocculation in mouse-brain virus suspensions at pH 4.0 to 5.5.

The effect of acidifying MM virus suspensions immediately before inoculation was also studied. Hammon and Izumi⁸ using the Armstrong mouse-adapted poliomyelitis virus and Wenner with the Lansing strain reported that the mortality rate for mice inoculated with mouse suspensions at pH 4.0 and 4.6 respectively, was higher than the death rate of animals given neutral or alkaline inocula. In order to determine whether the MM strain was affected by readjustment before inoculation, 10^{-3} and 10^{-5} dilutions of the virus were exposed to veronal-acetate buffers of pH 4, 6, and 8; the broth-salt control was at pH 7.2. After incubation for 1- and 24-hour intervals, a portion of each virus-buffer suspension was adjusted with 0.4% NaOH to about pH 7.5. These alkalized suspensions, as well as samples of the corresponding unadjusted virus-buffer mixtures, were inoculated into mice within an hour. The results indicated that readjustment to the favorable reaction range had no beneficial or potentiating action. In these experiments light was not excluded during the exposure periods.

In other tests, however, in which distilled water, physiologic saline, and buffer solutions were compared, precautions were taken to protect the test material from possible effects of light. Under these conditions, results with

TABLE III.
Survival of MM Virus in Acidified Distilled Water and Saline and in Veronal-acetate Buffer. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	pH		Period of exposure, days									
	1 day		7 days		1		7					
							Virus dilutions inoculated into mice					
	1 day	7 days	1 day	7 days	10-4	10-5	10-6	10-7	10-1	10-5	10-6	10-7
Distilled water + HCl	3.6	3.7	4/5	4/5	4/5	4/5	3/5*	1/5	5/5	0/5	2/5	0/5
" "	5.65	5.85	5/5	5/5	5/5	5/5	1/5	0/5	4/5	0/5	0/5	0/5
Distilled water	6.1	6.1	5/5	5/5	5/5	5/5	5/5	2/5	5/5	2/5	0/5	0/5
Saline + HCl	3.75	3.9	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Saline	6.1	6.25	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Veronal-acetate buffer	4.0	4.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
" "	6.0	6.0	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
" "	7.25	7.2	5/5	5/5	5/5	5/5	4/5	1/5	5/5	3/5*	0/5	0/5

Numerator = number of mice which died; denominator = number of mice inoculated. Test period = 7 days.

* 1 survivor paralyzed.

⁷ Wenner, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 104.

⁸ Hammon, W. M., and Izumi, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 579.

TABLE II. Survival of MM Virus in HCl, Phthalate, Phosphate, and Borate Buffers. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	Exp. No.	pH	Period of exposure, days						
			1		2				
			10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵	10 ⁻⁶	10 ⁻⁴	10 ⁻⁵
HCl	3	1.12	0/5	0/5	0/5				
Phthalate buffer	3	2.10	1/5	0/5	0/5				
	3	3.00	0/5	0/5	0/5				
	3	4.00	1/5	0/5	0/5				
Phosphate buffer	3	5.00	0/5						
	4	6.00		3/5	1/5	2/5	0/5	3/5	0/5
	4	7.01		5/5	5/5	5/5	5/5	5/5	5/5
Borate buffer	4	7.75		5/5	5/5	4/5	3/5	4/5	4/5
	4	7.71		4/5*	5/5	4/5	4/5	5/5	5/5
	4	8.75		5/5	4/5	5/5	5/5	5/5	5/5
Control (distilled water)	3	9.55		5/5	4/5	3/5	3/5	0/5	0/5
	3	7.01		4/5	3/5			5/5	5/5
	4	7.47		5/5	5/5	5/5*	3/5*	5/5	3/5

Numerator = number of mice which died; denominator = number of mice inoculated. Test period = 7 days.
 * 1 survivor paralyzed. 1 ± 0.05 .

concentration were taken into account. Inoculation of mice with virus-free buffers at the extremes of pH indicated no toxicity for the solutions *per se*. When streaked on blood-agar plates, the mixtures gave no growth.

Experimental Results. Tables I and II indicate the pH tolerance of MM virus in the different buffers at room temperature. These results and other data not presented here show that under the conditions of the experiment the virus when exposed at 10^{-4} dilution was stable in the neutral and alkaline solutions. There was a definite loss of activity within twenty-four hours in the veronal-acetate and phosphate buffers below pH 7. On the other hand, the virus survived for at least 2 days in glycooll and borate solutions at pH 9.95 and 9.55, respectively, but a decrease in activity was evident when tested on the 7th day. The veronal-acetate buffer, at approximately pH 7.8, contained infective virus in the 10^{-6} dilution after seven days at room temperature followed by refrigeration for 8 days. There was no significant difference between the phosphate and phthalate buffers at pH 6.0. However, the results did indicate that virus survival was somewhat better at around pH 8.0 in the borate than in the phosphate medium; at 10^{-4} dilution, active virus was present in the former but not in the latter buffer after 7 days at room temperature followed by 10 days at 4° - 6° C.

In a more detailed experiment, ten-fold virus dilutions from 10^{-2} through 10^{-6} or 10^{-7} in broth salt solution, were exposed to equal amounts of veronal-acetate buffers. Similar dilutions prepared with normal mouse brain had pH values of 4.35, 5.25, 6.35-6.45, 6.85-6.95, 7.75-7.8. After incubation for 1- and 7-day periods, suitable ten-fold dilutions were inoculated into mice. The results indicated, in general, a less deleterious effect of unfavorable pH in the more concentrated virus suspensions. The virus stability was definitely greater at pH 7.8 than at pH 6.9. In this experiment and in several others, there was occasional evidence of some virus survival at around pH 4.0 as compared to that at pH 5.0. While these results are suggestive of greater virus stability somewhere

rissoles implicated in a food poisoning outbreak.

Some success in producing experimental *Streptococcus faecalis* food poisoning in man is reported in the present paper.

Experimental. Studies with Kittens. Studies were carried out with 70 kittens in which evaporated milk containing 20-hour cultures (test animals) or evaporated milk alone (control animals) was fed. This line of investigation was found to be unsatisfactory and abandoned because it was noted that the controls occasionally developed diarrhea.

Studies with Human Subjects. All persons who served as subjects for these studies had undergone physical examination and gave no history of recent gastro-intestinal disturbance. Volunteers were given food which had been experimentally contaminated with *Streptococcus faecalis*.

Feeding with 20-hour Cultures. Attempts to produce experimental gastroenteritis with 20-hour cultures of *Streptococcus faecalis* were not fruitful. Twenty-one volunteers were fed 2.5 to 50 ml of 20-hour cultures of this streptococcus grown in milk or infusion broth. In no instance was a definite food poisoning syndrome provoked although 4 subjects complained of slight nausea which persisted in one individual for 24 hours. There were no such complaints among 11 volunteers who drank the vehicle without organisms. The reason for the failure of these 20-hour cultures to produce symptoms is not apparent.

Feeding with 5-hour Cultures. Four strains of *Streptococcus faecalis* were used. Three of these (A4, B3, JA2) had been isolated from human feces within 2 months of their use; the fourth, A34.1, had been isolated about one year previously from a can of evaporated milk implicated in an outbreak of gastroenteritis.

Three different foods were used; they were egg salad, custard, and sterile milk. Each food was prepared in average sized individual portions. The portions were inoculated with 5.0 ml of a 20-hour infusion broth culture, incubated at 37°C for 5 hours and then served. The egg salad portion consisted of

the amount needed for one sandwich, the custard portion of one cup, and the milk of 50 ml which was diluted with sterile milk to make up one glassful. Foods used for control purposes were prepared and incubated in the same manner as were the test foods except that no cultures were added to them.

All feedings were given shortly before noon. There was no marked difference in taste between the treated and untreated foods and the volunteers were unaware of the status of the food which each consumed. The volunteers limited their subsequent luncheon to lettuce and tomato sandwiches, tea or black coffee, and fruit.

When symptoms occurred after the experimental feeding the diagnosis was made by a physician who did not know the test status of the individual concerned.

Results and Discussion. The findings are recorded in Table I. It is noted that 6 of the subjects who ingested food contaminated with cultures of *Streptococcus faecalis* had definite symptoms of food poisoning, while a seventh had questionable ones. Nineteen volunteers who ate the same contaminated foods developed no symptoms, while 18 others who received the same foods but without addition of the specific bacteria, likewise, remained negative. Only 2 of the 4 strains used provoked symptoms of food poisoning. One of these was a recently isolated fecal strain and the other had been recovered about one year previously from food implicated in an outbreak. Thus 6, possibly 7, of 17 persons who ingested these strains became ill. Some of those who showed no symptoms ingested larger doses than others who did become ill. The production of symptoms in only a fraction of the subjects is paralleled by somewhat similar findings in some naturally occurring outbreaks of food poisoning apparently induced by *Streptococcus faecalis*.¹ The 2 strains which failed to produce symptoms were tested on a total of 9 persons.

The use of 5-hour cultures in this experiment was based on observations of the growth rates of these organisms in broth culture which indicated that good growth had occurred in that time and also, perhaps more

veronal-acetate buffers were similar to those previously obtained. In the range tested, from about pH 4.0 to pH 6.0, physiologic saline had an effect similar to that noted with veronal-acetate buffer solutions. On the other hand, the survival of virus in distilled water over the same range was apparently greater. Material diluted to 10^{-6} after exposure for 24 hours in a 10^{-4} dilution at about pH 4.0 was lethal for mice. After exposure for one week virus had survived in the 10^{-4} dilution close to pH 4.0 (Table III).

Summary. In various buffers, MM virus was stable at room temperature only at

neutral or alkaline reactions. When diluted virus was exposed at approximately pH 1.0 to neutrality the infectivity was diminished within twenty-four hours. There was a slower decrease in virus titer at pH 9 to 10. At pH 7 to 9, the virus was found to be stable for more than seven days. The deleterious effect of unfavorable pH was less for concentrated virus suspensions. MM virus was apparently more stable in distilled water acidified with hydrochloric or acetic acid than in acidified physiologic saline or in veronal-acetate buffer solutions of approximately the same pH value.

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Experimental Enterococcal Food Poisoning in Man.

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(Introduced by Ralph S. Muckenfuss.)

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Several outbreaks of food poisoning of a mild type in which enterococci were the predominant organisms isolated from the suspected foods, are described in a previous paper.¹ The literature on food poisoning caused by non-hemolytic streptococci is also discussed and it is pointed out that, in several instances in which the inculpatated organisms were closely identified, they were found to be enterococci.

The relatively small number of outbreaks reported as caused by *Streptococcus faecalis* is in sharp contrast to the widespread occurrence of this organism in nature.¹ Accordingly the studies reported in this paper were initiated in an attempt to determine whether *Streptococcus faecalis* can act as a food poisoning agent. They consisted of feeding experiments with *Streptococcus faecalis* employing kittens and human volunteers.

The literature contains the following references to experimental streptococcal food poisoning in man. Cary, Dack, and Meyers²

reported that a volunteer who had eaten parts of several sausages similar to others which had been implicated in an outbreak of mild gastroenteritis, developed nausea which increased for 24 hours, with two periods of vomiting and epigastric pain, followed by marked exhaustion and constipation. A green "pleomorphic" streptococcus whose species designation is not determinable from the description given was isolated from the sausage. Later Cary, Dack, and Davison³ reported abdominal distress, cramps, and diarrhea, in 5 of 7 volunteers who ingested "reasonable" doses of several strains of alpha streptococci which had been implicated in 2 outbreaks of food poisoning. On the other hand, Dolman⁴ reported failure to produce food poisoning in any of 4 volunteers each of whom had eaten a meat pie heavily infected with *Streptococcus viridans* which had been isolated from

² Cary, W. E., Dack, G. M., and Meyers, E., *Proc. Soc. Exp. Biol. and Med.*, 1931, **29**, 214.

³ Cary, W. E., Dack, G. M., and Davison, E., *J. Inf. Dis.*, 1938, **62**, 88.

⁴ Dolman, C. E., *Canad. J. P. H.*, 1943, **34**, 97.

¹ Buchbinder, L., Osler, A. G., and Steffen, G. I., *Pub. Health Rep.*, 1948, **63**, 109.

significantly, on the knowledge that incubation periods of such duration of suspected foods are frequently found in food poisoning outbreaks. It was found that 10 to 25 fold increases of initially large inoculums had taken place in the vehicle foods prior to ingestion.

It is of interest that the number of organisms which were found to produce experimental symptoms in this study is of the same order as that which Kelly and Dack⁵ found able to produce symptoms of gastroenteritis when a strain of *Staphylococcus aureus* was fed. These authors produced illness in one volunteer with 48×10^9 organisms and in another with 69.3×10^9 . In the present study the symptom producing dose of *Streptococcus faecalis* ranged from 22.7×10^9 to 148×10^9 organisms. This comparison is noteworthy since staphylococcal food poisoning is believed to be caused by an "enterotoxin" while Sherman, Gunsalus, and Bellamy⁶

suggest the possibility that the toxic principle of *Streptococcus faecalis* poisoning may be tyramine formed by the decarboxylation of tyrosine by this organism. Also perhaps worthy of comment is the fact that like staphylococcal food poisoning, streptococcal food poisoning usually has a short incubation period.

Summary. Symptoms of acute gastric or intestinal disturbance or of both were produced in 6, or possibly 7, of 26 human volunteers who ate foods in which strains of *Streptococcus faecalis* had grown for 5 hours.

Attempts to produce similar symptoms in man with 20-hour cultures were unsuccessful. Kittens were found to be unsatisfactory test animals in feeding experiments with inoculated condensed milk.

Conclusion. The experimental production of acute mild intestinal or gastric disturbance or both in man, tends to confirm the etiological role of *Streptococcus faecalis* in naturally occurring outbreaks of food poisoning.

We are indebted to Drs. William Birnkrant and Morris Greenberg of the Bureau of Preventable Diseases who conducted the physical examinations and diagnosed the illnesses which occurred.

⁵ Kelly, F. C., and Dack, G. M., *A. J. P. H.*, 1936, **26**, 1077.

⁶ Sherman, J. M., Gunsalus, I. C., and Bellamy, W. D., 57th Annual Report, N. Y. State Col. Agr., Cornell University Agr. Exp. Sta., 1944.

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Distribution and Nature of the "Antigen" Responsible for Experimental Meningoencephalomyelitis in the Guinea Pig.*

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Previous work has shown that a meningoencephalomyelitis, sometimes with foci of demyelination, may occur in monkeys, guinea pigs, and rabbits 2 to 9 weeks following the subcutaneous or intramuscular injection of brain tissue mixed with acid-fast

bacilli in a water-in-oil emulsion.¹⁻⁶ In con-

* Aided by a grant from the United States Public Health Service.

[†] The able assistance of Mr. Robert H. Purnell, Cornell University Medical College, is gratefully acknowledged.

¹ Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131 (preliminary report in *J. Bact.*, 1946, **51**, 53).

² Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117 (preliminary report in *Science*, 1946, **104**, 362).

³ Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neur.*, 1947, **6**, 333.

⁴ Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

⁵ Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

TABLE I.
Food Poisoning in Human Volunteers.
Ingestion of Milk, Custard, and Egg Salad Inoculated with *Streptococcus faecalis* and Incubated at 37°C for 5 hr.

	Cultures	Quantity of food	No. of organisms per g ($\times 10^6$)	Test		Control (food without culture)	
				No. of persons fed	No. of persons ill	No. of persons fed	No. of persons ill
Milk	A4, A34.1, B3	50 ml	454-990	3	1		
Custard	A4, A34.1, B3, JA2	92-94 g	168-1,050	12	2	A	0
Egg Salad	A4, A34.1, B3, JA2	41-87 g	740-2,100	11	4 (1?)	B, C D, E, F, G (?)	0 0 0
	Case	Culture	Dose	Symptoms			
Milk	A	A4	22.7 \times 109	Nausea and regurgitation which began after 2½ hours and lasted for 24 hours. Slight diarrhea after 9 hours.			
Custard	(B	A34.1	45 \times 109	Nausea, diarrhea and repeated vomiting which began 2 hours after feeding and persisted for 32 hours.			
	(C	A34.1	79.2 \times 109	Nausea, eruction and mild diarrhea. Symptoms began after 2 hours and persisted for 37 hours.			
Egg Salad	(D	A4	35.7 \times 109	Nausea and vomiting, dizziness and abdominal cramps which began after 2 hours and lasted for 30 hours.			
	(E	A34.1	107 \times 109	Nausea, abdominal cramps and one bout of vomiting which began after 2 hours and lasted for 2 hours.			
	(F	A4	148 \times 109	Diarrhea after 8 hours.			
	(G (?)	A34.1	99.6 \times 109	Nausea which began after 2 hours and lasted for 12 hours.			

gen" responsible for the meningoencephalomyelitis is a phosphatide-like material present in the white matter of the central nervous system. Its properties serve to differentiate it from 4 of the 5 haptens thought to be present in brain.⁷ "Neurokeratin"¹⁰ has been eliminated by previous workers;⁵ "protagon" and "sphingomyelin"¹¹ are included in the

non-phosphatide fractions tested in the present experiments and found to be inactive; and the hapten thought to be present in gray matter^{11,12} is obviously not necessary since it has now been found that white matter (optic nerve) contains the "antigen." Whether the "antigen" is the same as the "alcohol-soluble brain hapten"^{11,12,13} must await further studies.

¹⁰ Bailey, G. H., and Gardner, R. E., *Am. J. Hyg.*, 1942, **36**, 205.

¹¹ Schwab, E., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1936, **87**, 426.

¹² Reichner H., and Witebsky, E., *Ibid.*, 1934, **81**, 410.

¹³ Rudy, H., *Biochem. Z.*, 1933, **267**, 77.

16341

Thiouracil and Mammary Growth.*†

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There are a number of reports in the literature which would appear to indicate that the mouse and the rat may respond differently, as regards mammary development and responsiveness, during experimentally induced hypothyroidism.

Mixner and Turner¹ have reported an increased mammary responsiveness of ovariectomized female mice to estrogen and progesterone treatment when thyroxin was simultaneously administered, while a decreased responsiveness followed thyroidectomy. Mixner² later found that thiouracil administration had an effect similar to thyroidectomy in decreasing mammary responsiveness. Morris *et al.*, found considerable mammary atrophy

in virgin female mice fed thiourea³ and thiouracil⁴. Gardner⁵ observed mammary growth in male mice fed desiccated thyroid, but the effect was dependent upon intact testes.

From these reports it would appear that, in the mouse, experimentally induced mild hyperthyroidism is conducive to enhanced mammary responsiveness, while the opposite is true of hypothyroidism.

In the rat, however, Leonard and Reece,⁶ and Smithcors and Leonard⁷ have found that thyroidectomy of both male and female rats caused an enhanced development of the mammary gland. Thyroidectomy also increased the effectiveness of administered estrogen or testosterone propionate in stimulating mammary alveolar development. Chamorro^{8,9} has also reported that thyroid-

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1097.

† The diethylstilbestrol and its dimethyl ether used in this investigation were generously provided by Merck and Co., Rahway, N.J., and the thiouracil by Lederle Lab., Pearl River, N.Y.

¹ Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **31**, 345.

² Mixner, J. P., *J. Dairy Science*, 1947, **30**, 578.

³ Morris, H. P., Dubnik, C. S., and Dalton, A. J., *J. Nat. Cancer Inst.*, 1946, **7**, 159.

⁴ Morris, H. P., Dubnik, C. S., and Dalton, A. J., Exhibit Abstract, Fourth Internat. Cancer Res. Congress, Sept. 2-7, 1947, St. Louis.

⁵ Gardner, W. U., *Endocrinology*, 1942, **31**, 124.

⁶ Leonard, S. L., and Reece, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1941, **28**, 65.

⁷ Smithcors, J. F., and Leonard, S. L., *Endocrinology*, 1942, **31**, 454.

⁸ Chamorro, A., *C. R. Soc. Biol.*, 1946, **140**, 499.

firmary work in this laboratory an ascending paralysis has developed in slightly more than half of some 90 guinea pigs following the single subcutaneous injection of one cc of similar emulsions containing guinea pig, rabbit, human, or beef brain suspended 1:20 in 0.9% saline. The illness usually began 15 to 31 days after injection, although it was delayed as long as 74 days in one case; the animals usually died within 10 days, although some recovered and others remained paralyzed for one to 2 months. Histological examinations of brain and spinal cord removed from about 30 paralyzed guinea pigs in every case revealed a patchy or diffuse perivascular inflammation, predominantly lymphocytic, in the leptomeninges and entire central nervous system, most concentrated in the white matter. The evidence that this disease may represent an allergic reaction has been recently reviewed.⁷ Further experiments now to be described have shown that the "antigen" responsible for this experimental disease is present in certain phosphatide-like extracts of brain tissue, in a preparation of "purified brain lipids,"⁸ and in optic nerve, which contains only white matter.

To learn about the nature of the "antigen," various extracts of whole moist beef or human brain were made according to the outline of Page,⁸ using acetone, cold petrol ether, cold 95% alcohol, ether, and warm pyridine. In all, 5 different preparations of phosphatide-fractions were made, these being insoluble in acetone and soluble in cold petrol ether and ether; 4 of these were further fractionated into a lecithin-component (soluble in cold 95% alcohol) and a cephalin-portion (insoluble in cold 95% alcohol). Eight different preparations of other fractions of brain

were made. These included cholesterol and other substances repeatedly soluble in acetone; cerebrosides and sphingomyelin or a mixture of these referred to as "protagon,"⁹ which were insoluble in acetone, cold petrol ether, and cold 95% alcohol but soluble in pyridine warmed to 30°-45°C and in alcohol warmed to 80°C; and residual material which was insoluble in acetone, cold petrol ether, and warm pyridine. Each of these fractions was made up to an approximately 5% suspension in 0.9% saline; an emulsion was then made by mixing two parts of this suspension in a Waring blender with two parts of Bayol-F¹ containing 2.5 mg/cc heat-killed tubercle bacilli and one part of Falba.¹¹ Each emulsion was then tested by injecting one cc subcutaneously into each of 3 guinea pigs. Four of the 5 emulsions containing the phosphatide-fractions produced paralysis in 6 of 13 guinea pigs injected, whereas none of the 8 emulsions containing the other fractions produced any illness in 22 guinea pigs injected.

As a check on the lipid nature of the "antigen," an emulsion of "purified brain lipids"⁸ was made and injected into 5 guinea pigs. Nine days following a second injection 3 months later, one guinea pig developed a characteristic paraplegia. None of 5 guinea pigs similarly injected twice with an emulsion containing "purified phosphatidyl-serine"¹² became ill.

To learn about the distribution of the "antigen," optic nerves, which are composed only of white matter, were procured post-mortem from 2 human beings, care being taken to avoid the perichiasmal tissue. One of 3 guinea pigs injected with an emulsion made from this tissue became paralyzed.

In summary, it would seem that the "anti-

⁶ Morrison, L. R., *Arch. Neur. and Psych.*, 1947, **58**, 391.

⁷ Stevenson, L. D., and Alvord, E. C., Jr., *Am. J. Med.*, 1947, **3**, 614.

⁸ A preparation generously supplied by Dr. Jordi Folch-pi, McLean Hospital, Waverley, Mass., and said to contain practically all of the lipids present in whole brain and to be free of protein and carbohydrate.

⁸ Page, I. H., *Chemistry of the Brain*, Charles C. Thomas, Springfield, 1937, p. 54.

⁹ MacLean, H., and MacLean, I. S., *Lecithin and Allied Substances, the Lipins*, Longmans, Green & Co., Ltd., London, 1927, p. 125.

¹¹ A light paraffin oil, obtained through the courtesy of Mr. K. L. Patterson, Stanco, Inc., New York City.

An adsorption base said to be a mixture of oxycholesterine and cholesterines derived from lanolin (Pfaltz and Bauer, Inc., New York City).

¹² Also supplied by Dr. Folch-pi and described in *J. Biol. Chem.*, 1942, **146**, 35.

gen" responsible for the meningoencephalomyelitis is a phosphatide-like material present in the white matter of the central nervous system. Its properties serve to differentiate it from 4 of the 5 haptens thought to be present in brain.⁷ "Neurokeratin"¹⁰ has been eliminated by previous workers;⁵ "protagon" and "sphingomyelin"¹¹ are included in the

non-phosphatide fractions tested in the present experiments and found to be inactive; and the hapten thought to be present in gray matter^{11,12} is obviously not necessary since it has now been found that white matter (optic nerve) contains the "antigen." Whether the "antigen" is the same as the "alcohol-soluble brain hapten"^{11,12,13} must await further studies.

¹⁰ Bailey, G. H., and Gardner, R. E., *Am. J. Hyg.*, 1942, **36**, 205.

¹¹ Schwab, E., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1936, **87**, 426.

¹² Reichner H., and Witebsky, E., *Ibid.*, 1934, **81**, 410.

¹³ Rudy, H., *Biochem. Z.*, 1933, **267**, 77.

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Thiouracil and Mammary Growth.*†

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There are a number of reports in the literature which would appear to indicate that the mouse and the rat may respond differently, as regards mammary development and responsiveness, during experimentally induced hypothyroidism.

Mixner and Turner¹ have reported an increased mammary responsiveness of ovariectomized female mice to estrogen and progesterone treatment when thyroxin was simultaneously administered, while a decreased responsiveness followed thyroidectomy. Mixner² later found that thiouracil administration had an effect similar to thyroidectomy in decreasing mammary responsiveness. Morris *et al.*, found considerable mammary atrophy

in virgin female mice fed thiourea³ and thiouracil⁴. Gardner⁵ observed mammary growth in male mice fed desiccated thyroid, but the effect was dependent upon intact testes.

From these reports it would appear that, in the mouse, experimentally induced mild hyperthyroidism is conducive to enhanced mammary responsiveness, while the opposite is true of hypothyroidism.

In the rat, however, Leonard and Reece,⁶ and Smithcors and Leonard⁷ have found that thyroidectomy of both male and female rats caused an enhanced development of the mammary gland. Thyroidectomy also increased the effectiveness of administered estrogen or testosterone propionate in stimulating mammary alveolar development. Chamorro^{8,9} has also reported that thyroid-

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1097.

† The diethylstilbestrol and its dimethyl ether used in this investigation were generously provided by Merck and Co., Rahway, N.J., and the thiouracil by Lederle Lab., Pearl River, N.Y.

¹ Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **31**, 345.

² Mixner, J. P., *J. Dairy Science*, 1947, **30**, 578.

³ Morris, H. P., Dubnik, C. S., and Dalton, A. J., *J. Nat. Cancer Inst.*, 1946, **7**, 159.

⁴ Morris, H. P., Dubnik, C. S., and Dalton, A. J., Exhibit Abstract, Fourth Internat. Cancer Res. Congress, Sept. 2-7, 1947, St. Louis.

⁵ Gardner, W. U., *Endocrinology*, 1942, **31**, 124.

⁶ Leonard, S. L., and Reece, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1941, **28**, 65.

⁷ Smithcors, J. F., and Leonard, S. L., *Endocrinology*, 1942, **31**, 454.

⁸ Chamorro, A., *C. R. Soc. Biol.*, 1946, **140**, 499.

ectomy of rats caused mammary hypertrophy and augmented the mammary stimulating effect of pregnenolone. Smithcors¹⁰ has found that thiouracil treatment of rats did not of itself produce mammary alveolar development, but enhanced the mammary alveolar response to administered estrogen.

Unlike the situation in the mouse, these reports indicate that in the rat experimentally induced hypothyroidism is conducive to enhanced mammary gland growth and responsiveness.

With regard to hyperthyroidism in the rat, Weichert, Boyd, and Cohen¹¹ reported enhanced mammary development, but the effect was one of induced pseudopregnancy, and hence dependent upon intact ovaries.

Certain recent observations on thiouracil administration at this laboratory appear to fit into the above pattern.

Twenty-one young male albino rats were castrated and divided into three groups. Group 1 (6 rats) served as a control. About 10 days later Group 2 (8 rats) and Group 3 (7 rats) were started on daily injections of 10 μ g of diethylstilbestrol in oil. Group 3 had 0.1% thiouracil added to its feed. At the end of 21 days of this treatment all groups were sacrificed. Body weight, pituitary lactogen content and thyroid weight changes in these animals have been reported previously in connection with another experiment.¹²

The mammary glands of the control group showed small to medium sized duct systems with little or no alveolar development. Group 2 showed an enhanced state of mammary development, with additional alveolar development in most animals. Group 3 showed a striking advancement of mammary development over the control group, and a marked improvement over Group 2. Extensive alveolar development was present with good duct extension.

The mouse experiment involved 15 intact albino males. Five served as controls. Four were maintained for 6 weeks on a grain ration containing 1.23 mg of dimethyl ether of diethylstilbestrol per kilo of feed. This level of estrogen was known to promote good mammary duct extension in male mice. Six were maintained for 6 weeks on the same level of estrogen with 0.2% thiouracil added to the feed after the first week.

The estrogen treated animals showed good duct extension as compared to the controls, with some alveolar development in one animal. Unlike the results of the rat experiment, no difference in the response to estrogen could be detected in the estrogen and thiouracil treated mice.

The conditions of the rat and mouse experiments are unfortunately different in several respects such as dosage, duration, and mode of administration. However, taken together with previous reports, the present results are indicative of a difference in the effect of thiouracil upon mammary responsiveness in the rat and mouse.

With regard to the rat, there has been reported an effect of hyperthyroidism and hypothyroidism on vaginal sensitivity to estrogen. The effect appears to parallel that on mammary sensitivity to estrogen. Van Horn¹³ found that in 20 of 24 castrated female rats in a hyperthyroid condition, approximately 3 rat units of theelin were necessary to produce estrus. Langham and Gustavson¹⁴ found the vaginal rat unit of estrone to be 1.33 μ g for castrate controls and 2.5 μ g for castrates receiving thyroxin injections. Conversely thyroparathyroidectomized castrates required only 0.86 μ g. Thiourea administered in the drinking water caused a marked immediate decrease in vaginal sensitivity to estrone followed by a gradual increase, the rats becoming as sensitive after 56 days as thyroparathyroidectomized castrate rats.

It is possible that the same underlying mechanism is responsible for the effect of

⁹ Chamorro, A., *C. R. Soc. Biol.*, 1946, **140**, 721.

¹⁰ Smithcors, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 197.

¹¹ Weichert, C. K., Boyd, R. W., and Cohen, R. S., *Anat. Rec.*, 1934, **61**, 21.

¹² Meites, J., and Turner, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 488.

¹³ Van Horn, W. M., *Endocrinology*, 1933, **17**, 152.

¹⁴ Langham, W., and Gustavson, R. G., *Fed. Proc.*, 1946, **5**, 143.

hypothyroidism on the observed variations in the vaginal and mammary sensitivity of rats to estrogen.

The seemingly anomalous situation of thiouracil enhancing mammary growth response in the rat may have an analogy in body growth also. Astwood¹⁵ states that, although the prolonged administration of effective doses of thiouracil to rats causes an arrest of development and a retardation of growth, a small dose of thiouracil, given from the 21st day of life for a period of 9½ months, resulted in increased growth, including a gain in skeletal dimensions. The implication is that a mild degree of hypothyroidism is conducive to excessive growth in this species.

The authors are not familiar with any data indicating a similar relationship in the mouse.

Koger and Turner¹⁶ have studied the effect of experimentally induced hyperthyroidism in 4 species, including the rat and mouse. In the mouse it was found that although excessive hyperthyroidism inhibited body growth, a mild degree of hyperthyroidism consistently accelerated the growth rate. In rats however, mild hyperthyroidism had no such desirable effect, with the possible exception of a few animals that showed limited acceleration of growth.

It would appear from such experiments that the rat is normally secreting a level of thyroxin close to the upper limit of tolerance, and may actually be benefitted in some respects by a slight reduction in the normal

thyroxin secretion rate. If such is actually the case, this relationship would have wide implications in connection with the choice of experimental animals for certain types of investigation. It is known for instance that the normal rat is a poor animal for use in the assay of thyrotrophic hormone. Its thyroid gland is normally quite hyperplastic and relatively insensitive to further thyrotrophic stimulation.

That this relationship may not be true of all strains of rats is indicated by the work of Palmer *et al.*¹⁷ These authors were able, by selection of the progeny of a single pair of rats, to produce two strains which differed appreciably in their efficiency of food utilization. This difference was accompanied by a difference in the basal metabolic rate and in the effect of administered thyroid on skeletal length and on efficiency of food utilization. This was interpreted by the authors as indicating a possible difference in the thyroid secretion rate of the two strains.

The inheritance of varying rates of thyroxin secretion may be responsible in part for the marked differences in the growth rate and body size of the Yale and Wistar strains of albino rats.¹⁸

Conclusion. It would appear that there exist differences in the reaction of the mouse and rat to experimentally induced hypothyroidism and hyperthyroidism, possibly dependent upon the relative thyroxin secretion rates of the two species.

¹⁵ Astwood, E. B., *N. Y. Acad. Med., Harvey Lectures*, 1944-45, **40**, 195.

¹⁶ Koger, M., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 377, 1943.

¹⁷ Palmer, L. S., Kennedy, C., Calverley, C. E., Lohn, C., and Weswig, P. H., *Minn. Agr. Exp. Sta. Tech. Bul.* 176, 1946.

¹⁸ Harned, B. K., and Cole, V. V., *Endocrinology*, 1939, **25**, 689.

Effects of Electroshock Convulsions on Chronic Decorticated Cats.

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Masserman¹ has presented evidence that electroshock convulsions produce marked changes in the adaptive behavior of intact cats with experimental neuroses, comparable to the effects of electroshock therapy in man. In view of the evidence² that metrazol convulsions have excitant effects on autonomic centers of the hypothalamus, it was considered of value to investigate the possibility that electrically induced convulsions alter not only learned adaptive responses at the cortical level, but also non-learned, subcortically integrated responses which are closer to instinctive adaptive patterns. A study was therefore made of the effects of single and repeated electrically induced convulsions on the reactions of chronic decorticated cats to various stimuli, observations being limited to directly observable phenomena in the non-narcotized, non-curarized preparation.

In addition, this study afforded an opportunity to study the electroencephalographic patterns of electroshock convulsions in animals without neocortex. This was accomplished in terminal experiments on the decorticated cats after curarization.

Methods. Six cats were prepared by successive ablation of each cerebral hemisphere under nembutal anesthesia. Two operations were made, a week or more apart, under aseptic conditions without previous ligation of the carotid arteries. After recovery from anesthesia, all preparations exhibited the usual behavior and reactions of chronic decorticated cats and remained in good condition until terminal experiments were performed 29 to 147 days after completion of decortication. Observations were made on spontaneous behavior, posture, righting reflexes, licking reflexes, rectal temperature,

pulse and respiratory rates, and sham rage responses to handling and to nociceptive stimulation. To evoke nociceptive responses, a device was employed which permitted the quick application of known pressures from 1.0 to 10.0 kg to a segment of the tail for one second. With this apparatus the pressure in kg per cm² necessary to elicit a given response is a function of the area over which the pressure is applied. Hence, in this paper, all references to "pressure" are expressed in kilograms, understood to be applied to the specific jaw area of the instrument (1 cm²). Sham rage was also elicited in response to presumably non-nociceptive stimuli by holding the preparation by the loose skin around the back of the neck and alternately raising and lowering it rhythmically so that the hind footpads lightly touched the surface of the table on the downward movement. Control observations were made at intervals of 1 to 3 hours over a period of 8 hours on each of two successive days and before each experiment with electroshock convulsions.

Convulsions were produced by passing 200 to 450 milliamperes of 60 cycle A.C. for 0.2 to 0.3 sec. from vertex to palate. The electrode at the vertex consisted of a battery clip covered with saline paste attached to the scalp between the ears. The palate electrode consisted of a metal rod, one end of which was covered with gauze padding soaked in salt solution, and applied to the hard palate just behind the upper canine teeth. A commercial electroshock apparatus (Offner Type 735) was employed throughout. This device permits the delivery of a predetermined amperage regardless of variations in circuit resistance or size of the head. As will be shown below, the currents used appeared to exert no deleterious effects either physiologically or anatomically. From observations on conscious patients it is known that electroshock treatments are not painful.

¹ Masserman, J. H., and Jacques, M. G., *Am. J. Psychiat.*, 1947, **104**, 92.

² Gellhorn, E., *Am. J. Psychiat.*, 1941, **97**, 944.

Studies on the effects of electroshock convulsions were begun 7 to 37 days after completion of decortication in 5 of the preparations. The sixth was utilized only in the terminal experiment, 29 days after total decortication. After 48-hour observations on the effects of single electrically induced convulsions, 5 to 9 daily convulsions were induced, following which the preparations were observed during a 4- to 39-day period. One preparation was subjected to a second series of electroshock convulsions and observed for 7 days thereafter.

In terminal experiments carried out on 4 of the preparations, records were made of the electrical activity of the remaining brain immediately following passage of the current known previously to have induced convulsions regularly. This was accomplished by inserting, under ether, a tracheal cannula and screw electrodes into the calvarium and the body of the sphenoid bone (Fig. 1). The preparation was then immobilized by repeated intravenous injections of curare ("Intocostin," 0.3 to 0.5 cc at $\frac{1}{2}$ to $\frac{3}{4}$ hour intervals) and maintained on artificial respiration without anesthesia. Leads from the screw electrodes and one ear were connected to a capacity-

resistance coupled amplifier-oscillograph system with optical recording on moving bromide paper. In all experiments the leads to the amplifiers were disconnected before and during the passage of the convulsing current and replaced as soon as possible (3 to 5 seconds) afterward, with simultaneous removal of the electroshock leads, to reduce amplifier blocking.

After completion of these experiments the cats were sacrificed and the brain tissue was fixed *in situ* with formalin and removed for gross and microscopic study.*

Results. A. Motor Pattern of Electrically Induced Convulsions.

Immediately after electroshock the preparation assumed a posture of general semiflexion and for 15 to 20 seconds exhibited rapid rhythmic oscillatory movements of small amplitude involving the extremities, jaw and facial musculature. Frequently, several such paroxysms occurred, separated by quiescent periods lasting a few seconds. Finally, transient running movements of the hindlimbs appeared, after which all activity ceased for about 30 seconds. The preparation then righted itself or maintained the upright posture when so placed. During the seizure, apnea occurred, followed by transient hyperpnea. Occasionally, the preparation exhibited a burst of sham rage lasting 15 to 30 seconds, immediately after the seizure. One preparation circled for several minutes after each convulsion. With smaller currents greater variations were seen, such as sham rage without a preceding seizure or falling to one side in semiflexion without further motor activity. The motor pattern of the convulsions elicited by a given electroshock was remarkably constant for each preparation.

B. Effects of Single and Repeated Electrically Induced Convulsions.

Changes in temperature and pulse rate after single or repeated convulsions did not exceed spontaneous variations noted in control studies. No enduring impairment of righting re-



FIG. 1.

Cat No. 105 (chronic decorticate). X-ray of skull showing location of screw leads used for recording electrical activity of remaining brain in terminal experiments. Both screws are in the midline sagittal plane; the upper just penetrates the calvarium at the vertex and the lower extends to about 1 mm below the floor of the sella turcica in the body of the sphenoid bone.

* The neuropathological examinations were made by Dr. I. Mark Scheinker, Assistant Professor of Neuropathology, University of Cincinnati College of Medicine.

flexes occurred after single or repeated seizures. In 4 of the preparations, vigorous licking movements of the tongue could be induced by tactile stimulation of the perineal region. The licking appeared to be directed to any object nearest the tongue, e.g., the forepaws, the experimenter's hand, etc. This response was abolished for from $\frac{1}{2}$ to $3\frac{1}{2}$ hours after each convulsion in 3 of the cats. In the fourth preparation, no change occurred. No additional or cumulative effects were noted after daily convulsions.

In control observations, the threshold for sham rage induced by rhythmically raising the cat from a table varied from one such maneuver in one cat to no reaction after 50 trials in another. The day-to-day variations were also considerable, except in the cat with the lowest threshold, which always exhibited sham rage after one maneuver. The rage pattern thus evoked consisted of springing or lunging, boxing and scratching movements of the forelimbs, extrusion of the claws, lashing of the tail, retraction of the angle of the mouth, dilation of the pupil, retraction of the nictitating membrane and piloerection. Vocalization, spitting or hissing did not occur. After each electrically induced convulsion, such rage reactions were either unaffected or increased as indicated by a reduction in the number of maneuvers needed to elicit the response compared with the control value for that day. After completion of a series of daily electroshock convulsions, there seemed to be some intensification of the rage response to handling, but this could not be confirmed by the threshold values which were not altered beyond the range of variation in the control studies.

In control observations the threshold for sham rage induced by graded pressure on the tail varied from 3.0 to 7.0 kg from cat to cat; in each preparation daily variations of about 1.0 kg were observed. The pattern of reaction to such stimuli was remarkably constant in each cat but varied in detail from one to the other. In general, the responses consisted of vocalization, spitting and hissing, struggling, lashing of the tail, mydriasis and retraction of the nictitating membrane. After each elec-

trically induced convulsion, no reaction at all could be evoked even with maximum pressures of 10.0 kg, the limit of the instrument, for periods of from one to $2\frac{1}{2}$ hours, and the thresholds in most instances did not return to the control level before 4 hours—i.e., peak analgesic effects of over 50% to over 300% were observed. This was in marked contrast to the concomitant lowering of the rage threshold to handling described above. No significant changes occurred after completion of a series of daily electroshock convulsions.

C. Electroencephalographic Patterns of Electroshock Convulsions.

The electrical patterns of the seizures recorded in the terminal experiments varied in detail from one preparation to another, but the record illustrated in Fig. 2 A-C is fairly representative. After a single electroshock, several bursts of relatively high (75 to 100 microvolts) activity appeared, with frequencies varying from 2 to 21 per second. These paroxysms were separated by abruptly appearing silent intervals. In 3 of the experiments, a steady 15 to 18 per second rhythm appeared before cessation of the convulsive electrical discharge, which was followed by periodic isolated high voltage slow waves which were more prominent in tracings with a vertex lead. Such records could be obtained in each preparation several times by repeated electroshocks.

In places, a series of alternating fast and slow wave sequences was seen. Since in our laboratory typical "spike and dome" complexes have been observed in dogs following convulsant doses of morphine, we investigated the effects of this drug on the electrical patterns described above (Fig. 2 D-G). It will be noted that the slow and fast wave sequences after electroshock are more prominent in the morphinized preparation.

D. Neuropathologic Findings.

On removal of the remaining brains after terminal experiments, the dead space left by removal of the cerebral hemispheres was found to be occupied by fibrous connective tissue which bound the dura to the underlying brain structures. Coronal sections revealed no evidence of neocortex; varying portions of the caudate

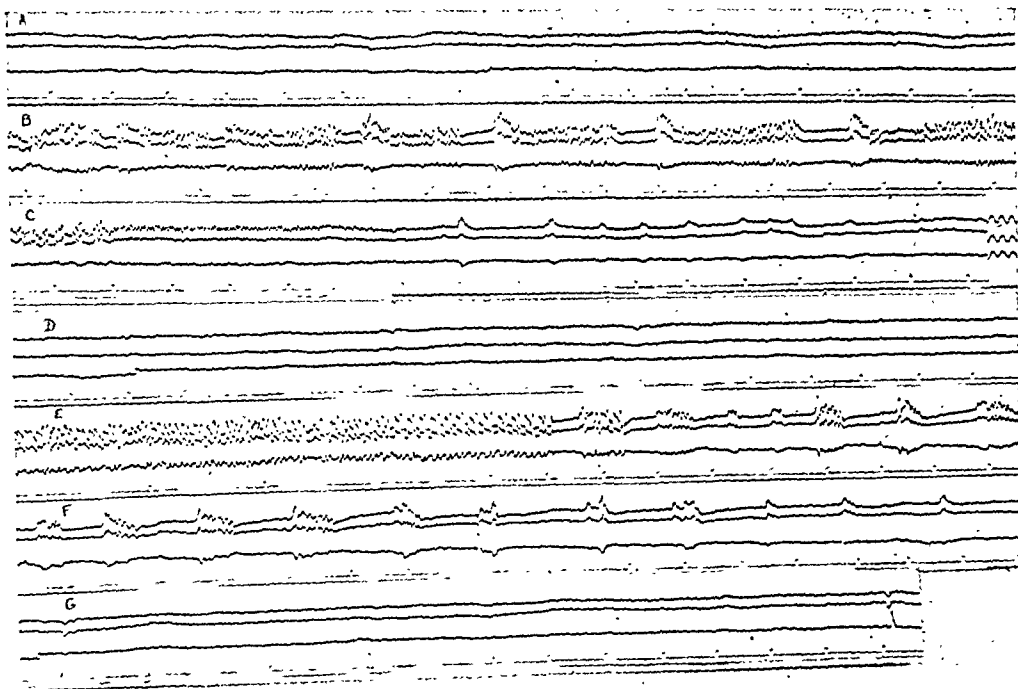


FIG. 2.

Cat No. 105 (chronic decorticate). Effects of electro-shock on electrical activity of remaining brain. Screw leads as in Fig. 1; clip lead on left ear. Preparation curarized and on artificial respiration. Time in seconds. Calibrations 23.8 microvolts peak-to-peak. In all records upper tracings are sphenoid to vertex, middle tracings sphenoid to ear, and lowest tracings vertex to ear. A, control. No significant electrical activity at this gain. B, 3 to 5 seconds after passing 450 milliamperes 60 cycle A.C. from vertex to palate for 0.3 sec. Note paroxysmal discharges of high voltage 6 to 21 per sec. waves originating in vicinity of the sphenoid lead. In places, a fast and slow wave occur in sequence, most often at the beginning of a paroxysm. C, continuation of preceding record. Note terminal regular 18 per sec. discharge of moderate voltage and random slow waves as record returns to control state. D, resting record 35 minutes after intravenous injection of morphine 20.0 mg per kg. No significant change from A. E, 3 to 5 sec. after electroshock as in B. Note paroxysmal discharges as in B, with greater tendency for fast and slow waves to occur in sequence. F and G, continuous with E. Note subsidence of electrical activity and return to control state.

nuclei, thalami and basal parts of the pyriform lobes were preserved. Microscopic study revealed evidence of degeneration with glial and connective tissue proliferation in the basal ganglia, especially in the periphery. In one specimen changes indicative of granular ependymitis were seen, and in another, evidence of mild subarachnoid hemorrhage. These changes did not differ in degree or kind from changes seen in the remaining brains of other chronic decorticated cats not subjected to electroshock or other convulsant agents in our laboratory.

Discussion. On comparison of our results with those of Masserman,¹ it is evident that,

although transitory changes did occur in the licking and sham rage reactions of the former, recovery was rapid and no enduring effects comparable to those in intact neurotic animals were observed. It would seem, therefore, that the long-enduring effects brought about by electroshock in Masserman's neurotic cats were due to changes involving the cerebral cortex. This inference is supported by the observations on the electroencephalographic changes in intact cats subjected to repeated electroshock convulsions, reported by Rubinstein and Kurland.²

² Rubinstein, H. S., and Kurland, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 348.

flexes occurred after single or repeated seizures. In 4 of the preparations, vigorous licking movements of the tongue could be induced by tactile stimulation of the perineal region. The licking appeared to be directed to any object nearest the tongue, e.g., the forepaws, the experimenter's hand, etc. This response was abolished for from $\frac{1}{2}$ to $3\frac{1}{2}$ hours after each convulsion in 3 of the cats. In the fourth preparation, no change occurred. No additional or cumulative effects were noted after daily convulsions.

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Cultivation of *Rickettsia prowazeki* in Dead Chick Embryos.

ESTHER RABINOWITZ, M. ASCHNER, AND N. GROSSOWICZ. (Introduced by L. Olitzki.)

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The criterion commonly used for the determination of the state of life or death of an organism is the heartbeat. Chick embryos in which the heart has ceased to function must be regarded as dead, and this assumption is supported by the fact that such embryos are indeed incapable of further development.

However, an egg containing a dead embryo is not necessarily devoid of living cells. It is known that the cells of an organism may survive after death for varying periods, depending on the external condition and on the type of tissue from which the cells were derived. Individual cells in fully developed tissues generally survive no more than a few hours if they remain in contact with the dead organism. However, in an embryo, in which cells are probably less interdependent, conditions need not be, and indeed are not, the same.

It has been demonstrated by Bucciante¹ that dead chick embryos may contain living cells for as long as 31 days after cessation of the heart beat, if the egg be maintained at 20°C after the death of the embryo; survival times at lower temperatures were even longer, up to 47 days.

It occurred to us that eggs containing surviving rather than developing tissue might constitute a favorable medium for the cultivation of *Rickettsia prowazeki* and related organisms.

According to Zinsser and Schoenbach² the rickettsiae develop best in cells with a low metabolic rate, and such a state may be assumed to occur in cells which survive without development for extended periods. However, the question was whether the cells would survive sufficiently long at temperatures

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Grodzinski,³ who examined the survival at 38°C of aorta tissue from dead chick embryos, reported a maximum survival time of 3 hours, which is, of course, insufficient for the cultivation of rickettsiae. However, this author tested only one type of tissue and worked with relatively well developed embryos, the youngest being 7 days old.

We tried using embryos which had been killed by chilling, after only 3 days development, and found that living cells could still be demonstrated in such material after much longer periods, even if the eggs had been maintained during this time at 37°C.

The following two cases may serve as illustrations: An egg containing a 3-day-old embryo was chilled at 4°C for 24 hours, and, after storage for 4 days at room temperature, was incubated for 10 days at 37°C. When the egg was opened after this period it contained a dead embryo which had not developed beyond its third day, yet living cells, mostly of epithelial character, grew out when fragments of the embryo or of its membrane were cultured in plasma clots.

In another case living cells were demonstrated by the same technique in an embryo which had been kept after death at room temperature for 7 days, and then for 16 days at 37°C. Results were practically the same in cases in which the embryos died spontaneously during the first 3 days of incubation as in those in which they were killed by exposure to cold.

R. prowazeki was inoculated into the yolk sac of eggs containing dead embryos and these were incubated for varying periods of time at 37°C. As expected, the rickettsiae multiplied abundantly under these conditions. It

¹ Bucciante, L., *Arch. exp. Zellforsch.*, 1931, 11, 397.

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³ Grodzinski, Z., *Arch. exp. Zellforsch.*, 1932, 12, 587.

The differences between the immediate effects of electroshock convulsions on sham rage reactions to nociceptive tail pressure and to handling are also of interest in connection with the report by Pisetsky⁴ of the disappearance of painful phantom limbs after electric shock therapy in a patient, who, though no longer complaining of pain, still displayed considerable effect. The observations on decorticated cats suggest that such analgesic effects may be partly subcortical in origin. However, since no cumulative or long-enduring effects were observed in decorticated cats it is inferred that the permanent disappearance of phantom limb pain in Pisetsky's subject was probably due to effects involving the cortex.

The striking features of the electrical activity recorded from sphenoid leads in chronic decorticated cats after electroshock are the recurrent paroxysmal hypersynchronous discharges, suggestive of "status epilepticus." The mixture of fast and slow frequencies during such paroxysms resembles the pattern seen in the clonic phases of convulsions in man, while the terminal steady 15 to 18 per second discharge is more like that occurring in tonic seizures. Also noteworthy, especially in the morphinized preparations, are the rhythmic fast and slow wave sequences which resemble to some extent "petit mal" spike and dome complexes. However, the "spike" frequencies in the decorticated preparations were not faster than 24 per second, perhaps differing in this respect from cortical seizure discharges in man. Further studies are in progress to determine more precisely the relationship of the subcortical convulsive electrical discharges to those recorded from the cortex and to the motor seizure patterns.

Summary. (1) The effects of single and repeated convulsions induced by electroshock were observed in 6 chronic decorticated cats. (2) The seizures were characterized by a posture of general semiflexion with small amplitude rapid rhythmic movements of the limbs, jaws and facial musculature, interrupted by one or more short quiescent periods, terminating in running movements after which a more prolonged quiescent phase preceded recovery. Apnea occurred during the seizures and was followed by transitory hyperpnea. (3) Body temperature and pulse rate were not affected significantly. Righting reflexes returned a few minutes after each seizure. Licking reflexes were abolished for from $\frac{1}{2}$ to $3\frac{1}{2}$ hours after each convulsion in 3 preparations. Sham rage in response to a non-nociceptive stimulus was unaffected or enhanced temporarily after each electroshock convulsion. Sham rage responses (chiefly facio-vocal) to nociceptive pressure stimuli applied to the tail were markedly reduced or abolished for from 1 to $2\frac{1}{2}$ hours after each electrically induced seizure. (4) No changes other than those noted after single electroshocks were noted after repeated electrically induced convulsions. (5) The electroencephalographic patterns of electroshock convulsions in the decorticated preparations were characterized by bursts of relatively high voltage 2 to 21 per second rhythms separated by short silent intervals, frequently terminating in a steady 15 to 18 per second discharge before cessation of electrical activity. Slow and fast wave sequences appeared at times during the paroxysmal discharges and were more prominent in previously morphinized preparations. (6) Gross and microscopic studies of the remaining brains after completion of the experiments revealed no changes which could be ascribed to the electric currents used to evoke convulsions.

⁴ Pisetsky, J. E., *Am. J. Psychiat.*, 1946, **102**, 599.

Cultivation of *Rickettsia prowazeki* in Dead Chick Embryos.

ESTHER RABINOWITZ, M. ASCHNER, AND N. GROSSOWICZ. (Introduced by L. Olitzki.)

From the Department of Hygiene and Bacteriology, Hebrew University.

The criterion commonly used for the determination of the state of life or death of an organism is the heartbeat. Chick embryos in which the heart has ceased to function must be regarded as dead, and this assumption is supported by the fact that such embryos are indeed incapable of further development.

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³ Grodzinski, Z., *Arch. exp. Zellforsch.*, 1932, 12, 587.

is difficult quantitatively to compare the growth of rickettsiae in dead embryos with that in living ones, as several factors obviously influence the final result. The maximum number of rickettsiae obtainable per egg is perhaps greater in eggs containing living embryos, since their yolk sacs are more extensive than those of eggs which have developed for only 3 days. However, the concentration of rickettsiae per cell in the infected cells of dead embryos may surpass that reached in living ones.

The growth period for rickettsiae in dead embryos may easily be extended to 16 days, or double the time customary in work with living embryos. Therefore even minute inocula have a chance to multiply significantly. This was determined in the following manner: 3-day-old dead embryos and 7-day-old living embryos were inoculated in parallel series with decreasing amounts of the same suspension of rickettsiae. Inocula too small to yield a positive smear after the usual 7-days' incubation in the living embryos yielded smears containing numerous rickettsiae after 14 days' incubation in the dead embryos.

Several advantages are inherent in our method. Eggs have to be incubated prior to use for only 3 days, and may then be stored for sometime at room temperature like ordinary culture media. This fact is especially convenient in field work, in which eggs may be inoculated on the spot and thereafter transported to the laboratory without particular regard to correct incubation temperature, humidity, or protection from vibration during transport, as is necessary when dealing with living eggs. Furthermore, this method is economical, since the percentage of eggs which have to be discarded on account of premature death of the embryo is, of course, considerably reduced.

Summary. 1. Chick embryos which were killed by chilling on the 3rd day of development and were thereafter maintained at 37°C for 16 days still contained living cells.

2. *Rickettsia prowazeki* multiplied abundantly in dead chick embryos which contained surviving cells.

3. The significance of these findings and some advantages inherent in this method are discussed.

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Sectioning Techniques for Electron Microscopy Using a Conventional Microtome.

DANIEL C. PEASE AND RICHARD F. BAKER.

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Sectioning biological material for use with the electron microscope poses special problems. Useful thicknesses can be measured only in fractions of a micron so that cutting becomes a major difficulty. Sections have to be examined *in vacuo*, so that the mode of drying is a matter for serious consideration.

The authors know of only 3 prior attempts to adapt more or less conventional histological techniques to the preparation of specimens for the electron microscope. Richards, Anderson and Hance¹ took embedded material and

faced the block in a standard microtome. They then changed the angle of the block and cut wedges which they hoped would taper into the fractional micron range. To a certain extent they were successful, but apparently were unable to find a completely satisfactory solution to embedding and mounting problems. Von Ardenne² employed essentially the same principle. Sjöstrand³ has also attempted

¹ Richards, G. A., Anderson, T. F., and Hance, R. T., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 148.

to cut thin sections in an unspecified manner. Interest then shifted to the development of high speed microtomes by O'Brien and McKinley⁴ and Fullam and Gessler.⁵ Their cutting edges, moving with tremendous linear speeds, have cut sufficiently thin sections for effective use. But the design and construction of the high speed microtome is a complex engineering problem, and it is certain that it will always be a very costly instrument, not usually available.

The possibility that standard histological techniques could be adapted was first suggested to the authors by Dr. F. Kiss of Hungary. In conversations he stated that he had often cut fractional micron sections for use with the light microscope. He implied that the size of the block was a critical factor, and that the material should be doubly embedded in collodion and paraffin. It now appears that his claim was fully justified, for we have consistently been able to cut sections 0.2 microns in thickness.

A Spencer rotary microtome model No. 820 is used, but altered simply so that the unit of advance is approximately 1/10 of the calibrated value. To accomplish this the angle of lever action is reduced 90% with the attachment shown in Fig. 1 (which is readily removable for standard use).

Our technique has been developed using rat liver perfused with 2% osmic acid in the manner of Claude and Fullam⁶ who have published micrographs of guinea pig liver sectioned with the high speed microtome. Small blocks of tissue (approximately one mm cubes) are run up through the alcohol series, into ether-alcohol, then into 3. 6. and finally 10% collodion (Mallinckrodt Parlodion) dissolved in ether-alcohol. The collodion is hardened in chloroform, and the blocks

transferred to xylol by way of carbol-xylol. They are then infiltrated with 65°C paraffin. The blocks are finally mounted rigidly in the microtome after being pared down to present a face about one mm square.

To cut 0.2 micron sections it is hardly necessary to say that the knife must be very sharp, but proper stropping is quite adequate. The optimum adjustment of the knife tilt is extremely critical, and even though alignment marks are lined up on the holder, minor adjustments are still necessary every time the knife is moved or replaced.

The sections are cut at moderate speeds, perhaps slightly faster than would ordinarily be used. They tend to rumple somewhat as they are cut. But single sections or short series can be picked up with a fine brush and transferred to a standard specimen screen for the electron microscope. Then, working with a dissecting microscope, one or two corners are tacked down to the screen by pressure with a needle. Gentle teasing with either the brush hairs or with needles will take out the major wrinkles. Finally the section is flattened and given many points of contact with the screen by gently stroking it with the brush, or by rubbing with the blunt polished end of a glass rod. A flattened section on a screen can be seen in Fig. 2.

The final preparation for the electron microscope involves the removal of part or all of the embedding material, with the hazards attendant upon the final evaporation of the solvents. Three methods have been devised, each of which has certain advantages, so that it is worthwhile to consider all.

Method 1. The simplest preparation is to extract the paraffin but leave the collodion in place. This is done by dropping xylol repeatedly on the specimen screen and thus flushing it. It is finally air dried. The collodion which remains is not a serious handicap to observation, at least at a magnification of $\times 5,000$. as it is in the form of a very fine mesh with low density. Since the specimen was impregnated with collodion first, presumably all parts of the section remain supported in place by the collodion during the drying, and in spite of the paraffin extraction.

² von Ardenne, M., *Z. Wiss. Mikroskopie*, 1939, 56, 8.

³ Sjöstrand, Fritiof, *Nature*, 1943, 151, 725.

⁴ O'Brien, H. C., and McKinley, G. M., *Science*, 1943, 98, 455.

⁵ Fullam, E. F., and Gessler, A. E., *Rev. Sci. Instr.*, 1946, 17, 23.

⁶ Claude, Albert, and Fullam, E. F., *J. Exp. Med.*, 1946, 83, 499.

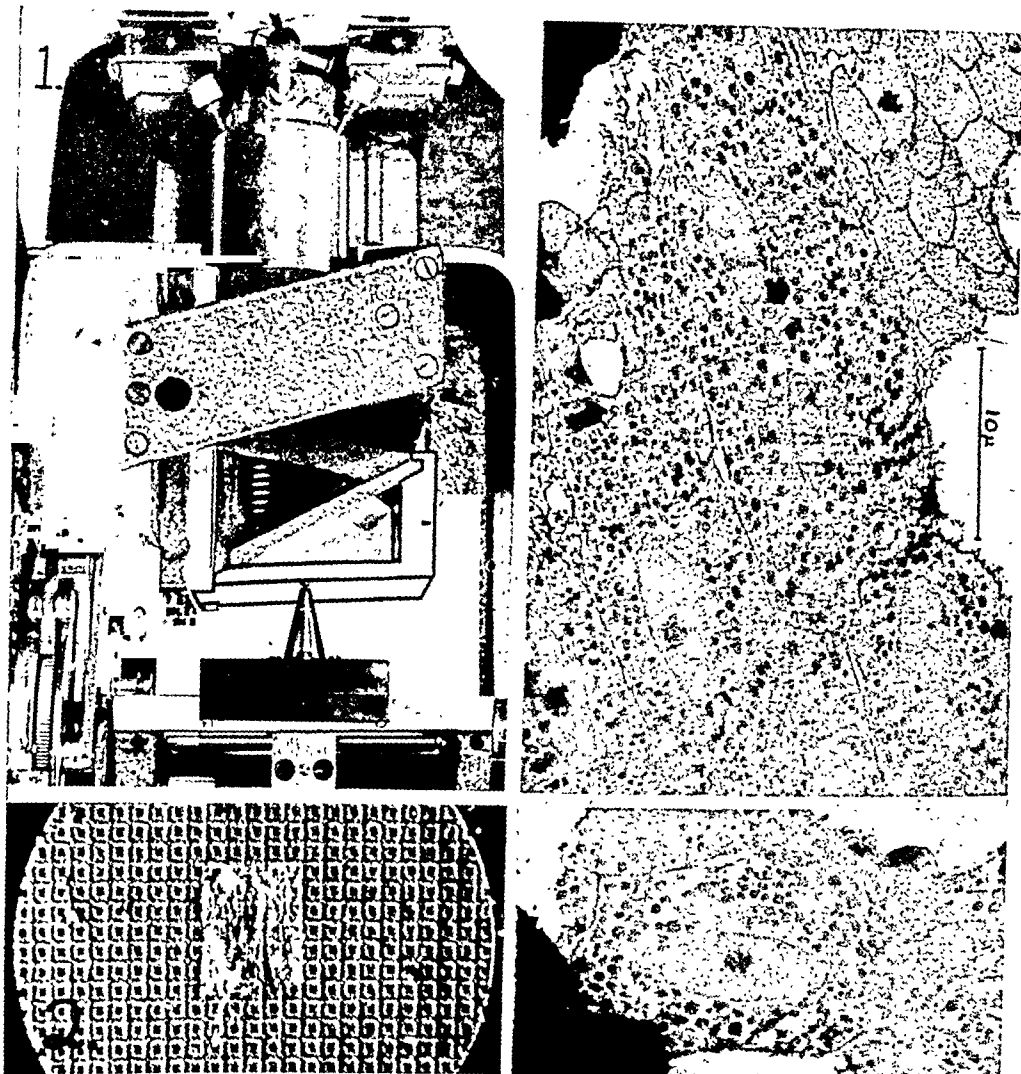


FIG. 1.

Vertical view of Spencer microtome modified by the outlined attachment to cut 0.1 micron.

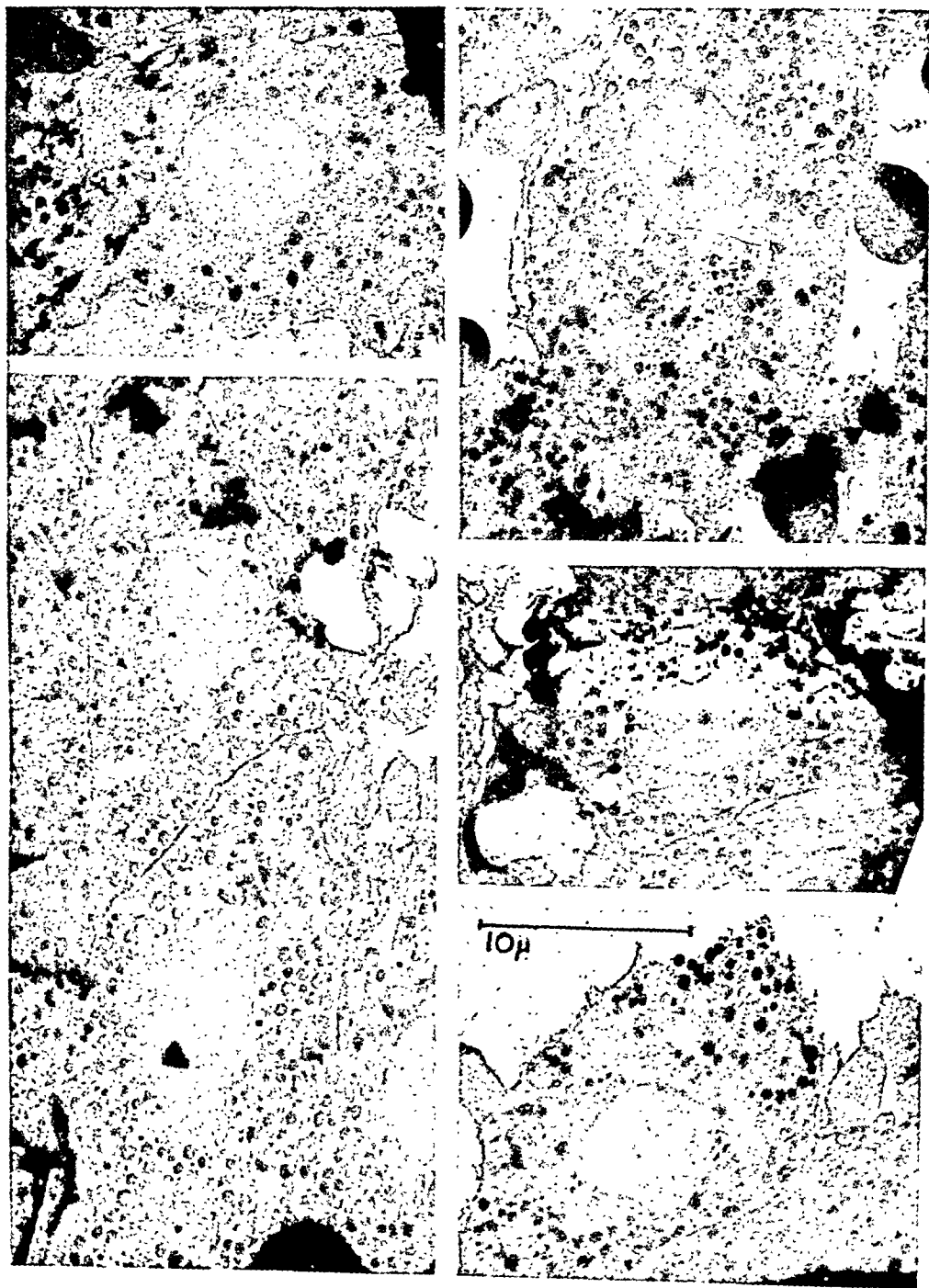
FIG. 2.

Enlarged view of a 0.2-micron section mounted on a standard 200-mesh specimen screen. The remaining figures show electron micrographs of rat liver sections cut at 0.2 microns. Cell walls, nuclei, nucleoli, mitochondria, bile capillaries, and blood sinusoids are visible. Sections of red blood cells appear in some sinusoids, although most were removed by the perfusion technique employed in the initial fixation with osmic acid.

Such specimens are very strong and resistant to electron bombardment.

Method 2. If the embedding media are to be entirely extracted surface tension may have serious distorting effects during the final drying of the solvents. However, if benzene is used as the final solvent it can be frozen

(5°C) and sublimed from the solid state thus minimizing the danger. Adherence of section to screen is none too good during this treatment. Best results are obtained by observing the following points. The screen, with the section tacked on, is inverted on a clean glass slide, and held down with a



needle. Amyl acetate is dropped on top and the screen flushed, thus removing the collodion. The section is very likely to become detached at this time, and surely will later if the screen is not now squeezed against the section once more. After rubbing the back of the screen with a blunt instrument the section sticks to the screen rather than to the glass, and the screen can be picked up and transferred to benzene which will remove the paraffin. Finally a drop of benzene surrounding the screen is frozen, and the benzene sublimed *in vacuo*. After such a total extraction the section is fragile and does not stand up well under the electron beam. This method is desirable particularly when the fine structure of a supporting medium might introduce uncertainty in the interpretation of the micrographs.

Method 3. In certain situations it presumably would be desirable to prepare serial sections. It is possible to do this, at least with short series, by varying the process as follows. Instead of tacking the sections to screens, they are flattened against a clean glass slide by teasing and brushing. Amyl acetate is flowed over them, followed by benzene, and finally amyl acetate once more. Then the slide is dipped in a 1% solution of collodion in amyl acetate and drained. The sections adhere to the glass throughout this process and are embedded in a thin film of collodion which is then dried. The slide is now dipped in distilled water thus floating the film off on the surface. Screens are then placed over the portions of the film containing sections, and are finally picked up and handled by usual techniques. Drying occurs only after the film is in place for support. A number of sections can be prepared simultaneously in this way. The paraffin is removed entirely, and the original network of collodion is replaced by a thin amorphous

film that is none the less quite tough.

The question of artefacts introduced by the method remains to be considered. The knife edge, as it cuts, has little tendency to compress the section. Some distortion may result from drying, but in most parts of a section is scarcely noticeable. The sections are not entirely uniform in thickness, but the variations are estimated to be usually less than .05 micron. Due to the non-uniformity one can find local areas that are probably less than 0.1 micron thick. Lines resulting from the chatter of the knife edge are sometimes present, and easily recognized.

The principal artefacts are certainly those of the original fixation as Claude and Fullam⁶ recognized in their work. In spite of very different treatments subsequent to fixation our micrographs are almost identical with theirs. Fixation artefacts become serious at magnifications above $\times 5000$ when it can be seen that much of the fine structure consists of precipitated fibers. The full potentialities of section cutting for the electron microscope, therefore, will be realized only after the development of superior methods of initial treatment.

Summary. Conventional histological techniques have been modified so that it is possible consistently to cut 0.2 micron sections for use with the electron microscope. The material must be doubly embedded in strong collodion and hard paraffin. The face of the block to be cut must be small, and the tilt of the knife must be precisely adjusted.

Procedures have been developed for partly or wholly removing the embedding media, and mounting the sections for the electron microscope.

Micrographs of rat liver sections show that the principal artefacts are due to fixation rather than subsequent treatments.

Hemolysis with Human Complement, Human Cells, and Tannic Acid: Application to Complement Fixation Test.*

LEWIS THOMAS AND JOHN L. PECK.†

From the Department of Pediatrics of the Johns Hopkins University School of Medicine and the Harriet Lane Home for Invalid Children, Johns Hopkins Hospital, Baltimore, Md.

It has long been known that tannic acid has an effect on erythrocytes which is analogous to the action of amboceptor; small concentrations of this substance produce agglutination of the cells but no hemolysis, while the addition of complement results in hemolysis.^{1,2} An investigation of this phenomenon was undertaken in this laboratory, as part of a study on the mechanism of action of complement. At first, considerable difficulty was encountered in demonstrating a consistent hemolytic effect with tannic acid and complement. It was found that the reaction was extremely sensitive to slight variations in the concentration of the sodium chloride solution employed as diluent for the reagents involved. When, however, the concentration of salt solution was fixed at 0.7%, rather than the usual 0.85%, it became possible to determine the optimal concentrations of tannic acid, complement and red cells for the reaction, with reproducible results. It was then learned that lysis of human erythrocytes could be brought about by fresh serum from the same or other individuals, in the presence of tannic acid. Moreover, accurate titrations of human complement could be made which yielded results comparable to those in the standard amboceptor-sheep cell system.

The present report deals with an application of the above findings to the complement fixation test, using human cells, human serum, and tannic acid as the indicator system. By

this method, estimations of specific antibody for lymphocytic choriomeningitis virus, streptococcus M₁G polysaccharide, and Wassermann antigen were performed, with results similar to those obtained by the standard complement fixation test.

Materials and Methods. *Diluent.* Sodium chloride dissolved in distilled water to make a 0.7% solution was employed as diluent for all reagents used in the test, in place of the usual physiological saline. The importance of using this diluent will be demonstrated below.

Cells. Blood obtained from a normal human subject was added to oxalate crystals. The cells so obtained were washed 3 times in physiological saline solution, and then suspended in sufficient 0.7% saline to make a cell concentration of 1%.

Complement. Whole human blood was allowed to clot at room temperature and then centrifuged at 2,000 r.p.m. for 10 minutes. The serum so obtained was kept in an ice bath. Fresh lots of cells and complement were prepared each day.

Tannic Acid. A single lot of tannic acid‡ was used in all the tests to be described. A 1% solution of tannic acid in 0.7% saline was made each day as a stock solution, and 2-fold dilutions from this were tested for the optimal tannic acid concentration by the method described below. In practice, it was found that a dilution of 1:16, or a 0.06% solution, was invariably suitable for producing hemolysis with complement.

Antigen-Antibody Systems. Three systems were employed in the tests to be reported. These were: (1) *Lymphocytic choriomeningitis virus antigen and antibody.* The antigen

* This work was supported by a grant from the Life Insurance Medical Research Fund.

† Senior Pediatric Fellow of the National Research Council.

¹ Reiner, von L., and Fischer, O., *Z. f. Immunitätsforsch.*, 1929, **61**, 317.

² Landsteiner, K., *The Specificity of Serological Reactions*, Harvard University Press, 1946.

‡ C. P. Crystalline Tannic Acid, lot No. 1, 1945, J. T. Baker Co., Phillipsburg, N.J.

consisted of guinea pig spleen soluble antigen, prepared by the method of Smadel, Baird and Wall.³ The antiserum was hyperimmune guinea pig serum, prepared after the method of these authors. (2) *Streptococcus MG polysaccharide antigen and antibody*.[§] The antigen consisted of a solution of purified polysaccharide from streptococcus MG,⁴ diluted 1/50,000 in 0.7% saline. Rabbit antiserum against this material was used as antibody. (3) *Wassermann antigen and antibody*.^{||} A standard preparation of Eagle beef-heart Wassermann antigen and several specimens of human luetic sera were used.

Each of the sera was heated for 30 minutes at 56°C before the test. Appropriate controls for the anticomplementary effect of both sera and antigens were included in each test.

Sensitization of Cells with Tannic Acid. Two parts of a 1% suspension of washed human red cells were mixed with one part of 0.06% tannic acid, in 0.7% saline. This mixture was prepared at least 15 minutes before being used, for reasons which will be described below.

Titration of Complement. Complement was titrated in the presence of the antigen used for the test. It was usually sufficient to set up 6 different amounts of complement, e.g. 0.07, 0.06, 0.05, 0.04, 0.03, and 0.02 cc of serum each contained in 0.2 cc of 0.7% saline. To each amount of complement were added 0.2 cc of the antigen, and 0.2 cc of 0.7% saline. These mixtures were then incubated at 37°C for 30 minutes, since this was the duration of fixation in all tests to be described. Following this period, 0.6 cc of the mixture of cells and tannic acid were added to each tube. The results were read after 30 minutes further incubation at 37°C. The endpoint, or the last tube in which complete lysis occurred,

was selected as one unit. For the complement fixation test, 1.5 units were employed.

The Test. The following amounts of the various reagents were used: Complement—0.2 cc (containing 1.5 units). Antigen—0.2 cc. Serum—0.2 cc. Sensitized cells—0.6 cc, consisting of 0.4 cc of 1% cells and 0.2 cc of 0.06% tannic acid.

Antiserum, antigen, and complement were added to the tubes in the order named. The tubes were then placed in a 37°C water bath and incubated for 30 minutes. At this time, 0.6 cc of sensitized cells were added to each tube, and the tubes reincubated for 30 minutes. Readings were then made of the degree of hemolysis. Fixation of complement was interpreted as occurring in those tubes showing no lysis, or one-plus lysis on a scale of 4+.

Results. The effect of small variations in the concentration of sodium chloride on the hemolytic reaction with human cells, human complement, and tannic acid is shown in Table I. In the experiment illustrated here, titrations of a sample of human complement were made in the presence of 0.06% tannic acid and 1% red cells. In each titration a different concentration of sodium chloride was used as diluent for the reagents involved. It will be seen that the greatest complement activity was evident in the row in which 0.7% sodium chloride was used as diluent, while the reaction was almost completely inhibited in concentrations of 0.85 and 0.9% sodium chloride.

The optimal concentration of tannic acid was found to be between 0.06 and 0.03%. When stronger solutions of tannic acid were used, for example a 0.1% solution, hemolysis did not occur. Solutions of less than 0.01% were inactive.

One percent suspensions of red cells yielded more consistent and reproducible results than 2% suspensions. With higher concentrations of red cells, incomplete hemolysis usually occurred regardless of the amounts of tannic acid or complement.

It was of great importance to sensitize the red cells with tannic acid at least 5 minutes before adding them to complement. If cells

§ Streptococcus MG polysaccharide and antiserum were kindly supplied by Dr. Frank L. Horsfall, Jr.

|| Wassermann antigen and luetic sera were kindly supplied by Dr. Thomas Farmer.

³ Smadel, J. E., Baird, R. D., and Wall, M. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 71.

⁴ Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., and Horsfall, F. L., Jr., *J. Clin. Inv.*, 1945, **24**, 227.

TABLE I.
Effect of Saline Concentration on Lysis of Human Red Cells by Human Complement and 0.06% Tannic Acid.

% of NaCl	Volume of Complement in 0.2 cc						Controls	
	.06	.05	.04	.03	.02	.01	.06 cc complement	.06% tannic acid
.60	++++*	++++	++++	++++	0	0	0	0
.65	++++	++++	++++	++++	0	0	0	0
.70	++++	++++	++++	++++	++	0	0	0
.75	++++	++++	++	+	0	0	0	0
.80	++++	++++	+	0	0	0	0	0
.85	++	+	0	0	0	0	0	0
.90	+	+	0	0	0	0	0	0

* +++++ Complete hemolysis.

+ Slight hemolysis.

0 No hemolysis.

TABLE II.
Complement Fixation with Lymphocytic Choriomeningitis, Streptococcus MG Polysaccharide, and Wassermann Antigens and Antibodies, Using Human Complement, Human Red Cells, and Tannic Acid.

Serum	Antigen	Initial serum dilution					Antigen Controls
		1/40	1/80	1/160	1/320	1/640	
Anti-MG	MG*	0†	0	0	0	++	++++
	LCM†	++++	++++	++++	++++	++++	++++
	NaCl	++++	++++	++++	++++	++++	++++
Anti-LCM	MG	++++	++++	++++	++++	++++	++++
	LCM	0	+	++	++++	++++	++++
	NaCl	++++	++++	++++	++++	++++	++++
Serum	Antigen	1/8	1/16	1/32	1/64	1/128	Antigen Controls
Luetic	Wassermann	0	0	0	0	+	++++
	NaCl	++++	++++	++++	++++	++++	++++
Normal	Wassermann	++++	++++	++++	++++	++++	++++
	NaCl	++++	++++	++++	++++	++++	++++

* Streptococcal MG polysaccharide 1/50,000.

† Lymphocytic choriomeningitis virus soluble antigen, from guinea pig spleen.

‡ Symbols as indicated in Table I.

and tannic acid were added separately to complement, little or no hemolysis occurred. If tannic acid and complement were allowed to stand together for a few minutes before the addition of cells, no hemolysis occurred. When cells and tannic acid had been together for 15 minutes, they could be used for the test for a period of at least 8 hours without deterioration.

The results of complement fixation tests with the three antigen-antibody systems studied are shown in Table II. It will be seen that the anti-streptococcus MG polysaccharide serum fixed complement in a dilution of 1:320 with the polysaccharide antigen, and showed no fixation with the unrelated lymphocytic-choriomeningitis antigen. The antiserum for the latter antigen, on the other

hand, fixed complement in a dilution of 1:80 and showed no fixation with the MG antigen. Normal rabbit serum showed no fixation with either antigen. The human luetic serum fixed complement with Wassermann antigen in a dilution of 1:128, while a normal human serum showed no fixation. Consistent results in the Wassermann test were more difficult to obtain than in the other antigen-antibody reactions described, because of the frequent appearance of an anticomplementary effect in Wassermann antigen with tannic acid-sensitized cells.

Each of the above sera and antigens were tested in 30-minute complement fixation tests by the usual method, using guinea pig complement, sheep cells, and rabbit amboceptor. The serum titers which were obtained

were substantially the same as those shown in Table II.

Comment. The observation of hemolysis of human cells by complement from the same individual, and the various conditions under which this reaction occurs, are of theoretical interest and suggest possible new approaches to the problem of complement's role in hemolysis. The complement fixation test itself may prove to be of practical value under some circumstances. There are certain advantages in a serological test which involves an homologous hemolytic system, instead of the 3 or more mammalian species which are represented in the usual complement fixation test. Moreover, the method makes it possible to do complement fixation tests under conditions where one or another of the usual reagents cannot be obtained.

There are certain limitations to the use of this method which should be pointed out. In Wassermann tests, the frequent finding of an anticomplementary effect of antigen presents a technical disadvantage. Moreover, the test is not feasible with antigens for which many human sera possess antibodies in low titer, such as influenza virus in allantoic fluid, since the complement employed for the

test may be partially fixed due to the presence of antibody in the same serum. For the same reason, it is probable that certain tissue antigens, with which human sera may react non-specifically in low dilution cannot be tested by this method. However, in those systems which lend themselves to the homologous test, the results appear to be clear-cut and are reproducible.

Summary. Human erythrocytes undergo lysis in the presence of tannic acid and human complement, even when cells and complement are obtained from the same blood sample. This reaction is dependent upon an optimal concentration of 0.7% sodium chloride, and an optimal concentration of between 0.06 and 0.03% tannic acid. When these substances are present in proper concentration, human complement can be titrated with reproducible results.

Specific fixation of human complement by three separate antigen-antibody systems has been demonstrated, employing homologous erythrocytes sensitized by tannic acid. Antibody titers determined by this method were comparable to those obtained in the standard complement fixation test.

16346

Effect of Chloromycetin on Experimental Infection with Psittacosis and Lymphogranuloma Venereum Viruses.

JOSEPH E. SMADEL AND ELIZABETH B. JACKSON.

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The results of the search for chemical and antibiotic substances which might be effective in the treatment of infections of viral and rickettsial origin have been reviewed recently

¹ Andrewes, C. H., King, H., and van den Ende, M., *J. Path. and Bact.*, 1943, **55**, 173.

² Kramer, S. D., Geer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

³ Jones, H., Rake, G., and Stearns, B., *J. Infect. Dis.*, 1945, **76**, 55.

by several groups of workers.¹⁻⁵ The contribution of Hamre and Rake⁵ is of especial interest as regards the current subject since it deals particularly with viruses of the psittacosis-lymphogranuloma group. The pres-

⁴ Cutting, W. C., Dreisbach, R. H., Halpern, R. M., Irwin, E. A., Jenkins, D. W., Proescher, F., and Tripi, H. B., *J. Immunol.*, 1947, **57**, 379.

⁵ Hamre, D., and Rake, G., *J. Infect. Dis.*, 1947, **81**, 175.

ent paper amplifies our earlier report⁶ on the use of Chloromycetin⁷ in experimental infections with psittacosis virus and summarizes our observations on the treatment of embryonated eggs and mice infected with the virus of lymphogranuloma venereum.

Materials and Methods. Virus material. The 6-BC and P-4 strains of psittacosis were employed in this work. The former, isolated in 1941 by Dr. K. F. Meyer from a parakeet, was obtained from Dr. E. Lennette as lyophilized yolk sac material; it is well adapted to growth in embryonated eggs and is lethal for mice when inoculated by either the intracerebral or the intraperitoneal route. The latter, isolated in 1942 from a sick pigeon,⁸ also grows profusely in the yolk sacs of embryonated eggs but is lethal for mice only when inoculated intracerebrally. The L. A. strain of lymphogranuloma venereum used in the present experiments was obtained in 1943 from the inguinal gland of a patient;⁹ it has been maintained since in this laboratory by passage in the yolk sacs of embryonated eggs or brains of mice.

Preparations of the 3 agents used in the present work consisted of 20% suspensions of infected yolk sac tissue triturated in sterile Difco skimmed milk media pH 7.2; these were cleared of large particles by light centrifugation and stored in 2.0 ml amounts in rubber stoppered tubes at -70°C . The infectivity of each of the stored suspensions was determined by titration in embryonated eggs inoculated by the yolk sac route. In addition, each suspension was titrated intracerebrally in mice, and the preparation containing the 6-BC strain was also titrated intraperitoneally in mice. Titers were calculated by the 50% end point method.¹⁰

Chemotherapy experiments in eggs. The

⁶ Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

⁷ Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

⁸ Smadel, J. E., Wall, M. J., and Gregg, A., *J. Exp. Med.*, 1943, **78**, 189.

⁹ Zarafonitis, C. J. D., *New England J. Med.*, 1944, **230**, 567.

procedure developed in this laboratory for testing the efficacy of drugs on the growth of rickettsial organisms in embryonated eggs¹¹ was followed in the current studies. In this technique, groups of 24 embryonated eggs which had been incubated for 7 days were inoculated by the yolk sac route with 0.1 or 0.2 ml of a solution containing the desired amount of drug; the control group received 0.2 ml of buffered saline. Then all the embryonated eggs were again injected into their yolk sacs with 0.2 ml of that dilution of standard virus suspension which would be expected to kill the majority of the embryos in the control group in 4 to 5 days. Usually 30 to 45 minutes elapsed from the time an embryo was treated with drug until it received the infectious inoculum. In certain tests therapy was delayed for 24 to 48 hours after infection. Eggs were kept from the time of setting and throughout the experiment in an incubator regulated to 37°C . Following inoculation they were candled daily and the time of death of the embryos recorded. Deaths occurring in the first 2 days were not counted in the results. The mean day of death of embryos in each group was calculated. An estimate of the efficacy of the drug was obtained by subtracting the mean value of the control group from that of the treated group; this gave a figure which was taken as the average prolongation of life of the treated eggs. The methods for statistical analysis of the present data were the same as those used previously.¹¹ Dr. Ross L. Gauld performed these calculations for us.

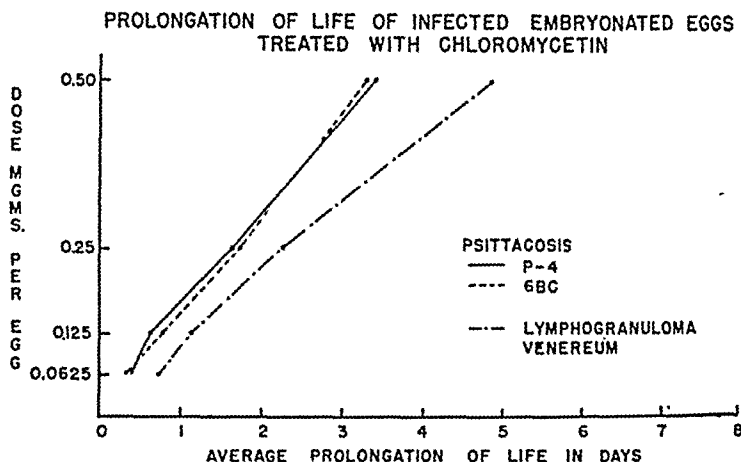
Chemotherapy experiments in mice. Swiss mice of the Bagg strain, weighing 14-18 g were used in the present experiments. Mice infected by the intracerebral or intraperitoneal route received 0.03 ml or 0.2 ml, respectively, of an appropriate dilution of standard frozen virus suspension. Chloromycetin was administered to mice by mouth or injected intra-

¹⁰ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

¹¹ Smadel, J. E., Jackson, E. B., and Gauld, R. L., *J. Immunol.*, 1947, **57**, 273.

¹² Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53**, 251.

FIG. 1



peritoneally. When the former route was used, sufficient drug was dissolved in distilled water so that each 1.0 ml of solution contained $\frac{1}{2}$ the desired daily dose for a mouse. Daily at 5:00 p. m. each group of 10 mice was offered 20 ml of such solutions in a drinking bottle. This amount was usually consumed by morning unless the mice were moribund. During the day the animals were offered tap water. For therapy by the intra-peritoneal route sufficient drug was dissolved in physiological saline solution so that each 1.0 ml contained the desired daily dose; this was given each morning at 9:00 a. m.

The lethal titers of the virus suspensions were calculated by the 50% end point method¹⁰ for each set of control mice, and where feasible, for the groups of drug-treated mice.

Results. Prolongation of life of infected embryos treated with varying doses of drug and at different times. Fig. 1 summarizes experiments in which doses of drug varying from 0.0625 mg to 0.5 mg were injected into the yolk sac just prior to infection with several hundred M.L.D.'s of one of the 3 agents. In this figure the average prolongation of life of the treated groups expressed in days is plotted against the amounts of drug given. The values for prolongation of life obtained in tests with doses of 0.0625 mg and above were statistically significant. It is apparent that a direct relationship exists between the amounts of drug employed and the pro-

longation of life of the treated embryos. Furthermore, the results obtained with the 2 strains of psittacosis virus are essentially identical. In this method of testing, lymphogranuloma virus would appear to be slightly more susceptible to chemoprophylaxis with the drug than psittacosis virus.

Chloromycetin has a chemotherapeutic effect in infected embryos even when treatment is delayed for 24 or 48 hours. The results obtained in tests with the P-4 strain of psittacosis are illustrated in Table I; these are typical of the data obtained with each of the 3 agents. The prolongation of life in the group treated 24 hours after infection was, in each instance, slightly greater than that obtained when treatment was given $\frac{1}{2}$ hour before infection. While the values for these differences in some of the 5 experiments with the 3 agents were such as might have occurred by chance, the fact that they all trend in one direction is significant. In the experiment illustrated, and in those with the other strains, the effect induced by treatment given 48 hours after infection approached that recorded for the group treated just prior to infection. In another experiment embryos were infected with approximately 1,000,000 M.L.D.'s of the 6-BC virus. The average day of death in the control group in this test was 3.1 days. Even when injection of the drug was delayed for 48 hours there was still a significant prolongation of life of

TABLE I.
Chloromycetin in Experimental Psittacosis in Eggs (Infecting Dose 500 MLD's of P-4 Strain).

No. of eggs in group	Drug treatment		Mean day of death	Prolongation of life (days)
	mg/egg	Time given (in hours)		
24	None		4.83	
24	0.5	½ Pre	8.13	3.30
24	0.5	24 Post	8.77	3.94
24	0.5	48 Post	7.64	2.81

TABLE II.
Chloromycetin in Experimental Psittacosis in Mice (Infecting Dose 80 MLD's of 6-BC Strain Intraperitoneally).

Route	Drug treatment		Dilution of infectious inoculum					
	mg/day mouse	Day begun						
			10-5	10-6	10-7	10-8	10-9	10-10
	None		10/10	7/10	8/10	6/10	2/10	0/10
I.P.	2.5	1 Post		1/10				
	2.5	3 "		1/10				
	2.5	6 "		1/10				
	1.5	1 "		0/10				
	1.5	3 "		2/10				
	1.5	6 "		1/10				
	0.75	1 "		0/10				
	0.75	3 "		4/10				
	0.75	6 "		7/10				
Fed	5.0	1 Pre		0/10				

TABLE III.
Chloromycetin in Mice Infected by Different Routes with 6-BC Strain of Psittacosis.

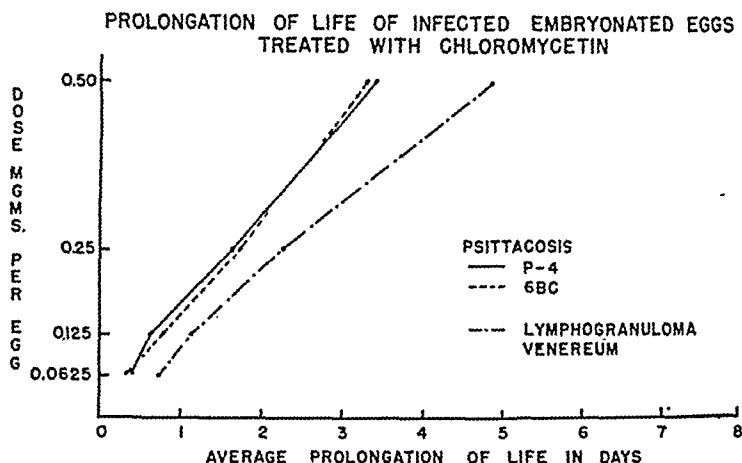
Exp.	Drug treatment			Route	Infectious inoculum								Titer
	Route	mg/day mouse	Day begun		Dilution								
					10-3	10-4	10-5	10-6	10-7	10-8	10-9	10-10	
1a		None		I.P.	10/10	10/10	10/10	6/8	8/10	6/10	5/10	0/10	10-8.3
	I.P.	2.5	1 post	0.2 cc	3/10	0/9	2/9	0/8	0/9				<10-3
	Fed	5.0	1 pre		5/10	2/10	2/10	0/8	0/10				10-3.4
1b		None		I.C.	10/10	10/10	10/10	10/10	10/10	10/10	5/10	0/10	10-9.0
	I.P.	2.5	3 hours pre	0.03 cc				10/10	10/10	10/10	4/10		10-8.8
	Fed	5.0	1 pre					10/10	9/10	9/10	2/10		10-8.5

the treated embryos, *i.e.*, 1.5 days.

Effect of Chloromycetin in mice infected by different routes. Mice infected by the intraperitoneal route with approximately 80 M.L.D.'s of the 6-BC strain of psittacosis were treated with Chloromycetin by the intraperitoneal or oral routes. Data from such an experiment are illustrated in Table II. In this and subsequent experiments the anti-

biotic was given to the mice in each test group from the time indicated until death occurred or until the 12th day after inoculation of the infectious material; treated and control mice were observed until the 21st day when the experiment was terminated. Seven of the 10 control mice which received the same concentration of challenge virus used to infect the treated mice died between the 6th and 10th

FIG. 1



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Discussion. Penicillin and drugs of the sulfonamide series have a therapeutic effect on experimental infections caused by a number of agents of the psittacosis-lymphogranuloma group. Furthermore, these substances have been employed in the treatment of patients with lymphogranuloma venereum or psittacosis (see ³). The present data obtained in infected embryonated eggs and mice treated

with Chloromycetin suggest that the new antibiotic possesses about the same degree of activity as that displayed by penicillin and sulfadiazine in similar types of studies.^{5,12} It is of interest that none of the drugs are really effective in treatment of mice infected by the intracerebral route with these agents.

Conclusion. Chloromycetin possesses considerable therapeutic activity in embryonated eggs and mice infected with the viruses of psittacosis or lymphogranuloma venereum. This activity is comparable in amount to that demonstrated by others for sulfadiazine and penicillin tested under similar conditions.

16347 P

Inhibition of Multiplication of Influenza Virus by Tannic Acid.*

ROBERT H. GREEN. (Introduced by Francis G. Blake.)

From the Department of Internal Medicine, Yale University School of Medicine, and the Medical Service of the New Haven Hospital, New Haven, Conn.

Recently it has been reported¹⁻⁸ that various substances inhibit multiplication of one or more viruses of the mumps-influenza group as well as the pneumonia virus of mice. In some instances these substances inhibit both virus multiplication and virus hemagglutination. Moreover, of considerable interest is

the fact that some of these substances are themselves capable of agglutinating erythrocytes. For the most part, when tested *in vitro* they exert little or no direct effect upon the viruses against which they are active *in vivo*. With the exception of the enzyme described by Burnet,⁸ which apparently acts upon the receptor substance of the cell, the mode of inhibitory action and the relationship of inhibition of hemagglutination to that of multiplication are obscure.

The fact that tannin agglutinates erythrocytes has been known for some time.⁹ During the course of studies on hemagglutination it was observed that tannic acid in concentrations as low as 45 μ g per cc agglutinates chicken erythrocytes. Subsequent investigation showed that smaller amounts of tannic acid actually inhibit the agglutination of chicken erythrocytes by influenza A virus. Furthermore, tannic acid inhibits the multiplication of influenza A virus *in vivo* and inactivates the virus *in vitro*.

* Aided by a grant from the United States Public Health Service.

¹ Wheeler, A. H., and Nungester, W. J., *Science*, 1944, **100**, 523.

² Green, R. H., Rasmussen, A. F., Jr., and Smadel, J. E., *Pub. Health Rep., U. S. P. H. S.*, 1946, **61**, 1401.

³ Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, **85**, 623.

⁴ Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

⁵ Liebmman, A. J., Perlstein, D., and Snyder, G. A., *J. Bact.*, 1947, **54**, 63.

⁶ Rubin, B. A., and Giarman, N. J., *Yale J. Biol. and Med.*, 1947, **19**, 1017.

⁷ Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 99.

⁸ Burnet, F. M., *The Lancet*, 1948, **254**, 7.

⁹ Freund, J., *J. Immunol.*, 1931, **21**, 127.

days. It is apparent from the tabular data that the daily intraperitoneal administration of 1.5 mg of Chloromycetin beginning 6 days after infection was effective since only one of the 10 mice in the group died. Half this amount of drug was ineffective when treatment was begun on the sixth day but did protect the entire group when given from the first day after infection. Finally, the data in Table II show that all mice which received approximately 5.0 mg of drug per day by mouth survived the infection.

A series of experiments was next performed in which mice were infected by the intracerebral route with the P-4 strain of psittacosis or the L. A. strain of lymphogranuloma venereum virus and treated with Chloromycetin either by mouth or by intraperitoneal injection. In none of these experiments was a beneficial chemotherapeutic effect demonstrated. The question then arose as to whether Chloromycetin would under any circumstance protect against intracerebral infection with a member of the psittacosis-lymphogranuloma group. The 6-BC strain of psittacosis was chosen for the next experiment because it is lethal when given either intraperitoneally or intracerebrally; this is in contrast to the P-4 strain of psittacosis and the lymphogranuloma virus which are lethal only when given by the intracerebral route.

In the experiment summarized in Table III, groups of mice were injected by one of these 2 routes with serial 10-fold dilutions of the 6-BC suspension and treated with Chloromycetin by daily injection or by feeding. It is apparent from the data presented in this table that treated mice survived inoculation with enormous amounts of virus injected by the intraperitoneal route but displayed no resistance to intracerebral inoculation of the virus.

Effect of Chloromycetin on the virus in vitro and in vivo. Chloromycetin possesses little or no direct virucidal action against the agent of psittacosis. This was demonstrated in an experiment in which 2 portions of a 10^{-5} dilution of a standard suspension of 6-BC strain were prepared; one contained the virus in ordinary milk diluent whereas in

the other the virus was suspended in milk media in which was dissolved 2.5 mg of drug per ml. These 2 portions were held at room temperature for 2 hours and then titrated in embryonated eggs. The lethal titers were $10^{-0.6}$ and $10^{-10.3}$, respectively. Furthermore, the average day of death of the embryos inoculated with dilutions of the control and drug treated suspensions of virus were essentially the same. As was to be expected the lives of the embryos which received the original virus suspension containing 2.5 mg of drug per ml were prolonged.

In the present experiments none of the embryonated eggs inoculated with even a few hundred M.L.D.'s of virus survived to the time for hatching even when treated with 0.5 mg of drug. Yolk sac tissues from moribund treated eggs were as rich in elementary bodies as were those from controls.

In the experiment summarized in Table II, practically all of the mice which survived when treatment was begun later than the first day after infection developed obvious signs of disease. Those least affected showed ascites but otherwise appeared fairly healthy. No apparent illness was noted among the mice given the drug by mouth beginning the day before infection. In this experiment the spleens from 2 animals of each treated group in which all mice survived were tested for virus by passage to normal mice; each suspension of splenic tissue contained active virus. Furthermore, the remaining animals in these groups resisted an intraperitoneal challenge inoculation containing several hundred M.L.D.'s of the 6-BC strain. Thus, infection was not prevented in mice treated prophylactically nor were the tissues freed of virus when the drug was used for chemotherapy.

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infective titers of the pooled hearts' bloods determined in mice were as follows: controls, $10^{-2.5}$; group treated with injections of 2.5 mg of drug, $10^{-1.3}$; group fed 5 mg of drug, less than 10^{-1} (1 of 5 mice receiving a 10^{-1} dilution of blood succumbed).

Discussion. Penicillin and drugs of the sulfonamide series have a therapeutic effect on experimental infections caused by a number of agents of the psittacosis-lymphogranuloma group. Furthermore, these substances have been employed in the treatment of patients with lymphogranuloma venereum or psittacosis (see ⁷). The present data obtained in infected embryonated eggs and mice treated

with Chloromycetin suggest that the new antibiotic possesses about the same degree of activity as that displayed by penicillin and sulfadiazine in similar types of studies.^{5,12} It is of interest that none of the drugs are really effective in treatment of mice infected by the intracerebral route with these agents.

Conclusion. Chloromycetin possesses considerable therapeutic activity in embryonated eggs and mice infected with the viruses of psittacosis or lymphogranuloma venereum. This activity is comparable in amount to that demonstrated by others for sulfadiazine and penicillin tested under similar conditions.

16347 P

Inhibition of Multiplication of Influenza Virus by Tannic Acid.*

ROBERT H. GREEN. (Introduced by Francis G. Blake.)

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Recently it has been reported¹⁻⁵ that various substances inhibit multiplication of one or more viruses of the mumps-influenza group as well as the pneumonia virus of mice. In some instances these substances inhibit both virus multiplication and virus hemagglutination. Moreover, of considerable interest is

the fact that some of these substances are themselves capable of agglutinating erythrocytes. For the most part, when tested *in vitro* they exert little or no direct effect upon the viruses against which they are active *in vivo*. With the exception of the enzyme described by Burnet,⁶ which apparently acts upon the receptor substance of the cell, the mode of inhibitory action and the relationship of inhibition of hemagglutination to that of multiplication are obscure.

The fact that tannin agglutinates erythrocytes has been known for some time.⁹ During the course of studies on hemagglutination it was observed that tannic acid in concentrations as low as 45 μ g per cc agglutinates chicken erythrocytes. Subsequent investigation showed that smaller amounts of tannic acid actually inhibit the agglutination of chicken erythrocytes by influenza A virus. Furthermore, tannic acid inhibits the multiplication of influenza A virus *in vivo* and inactivates the virus *in vitro*.

* Aided by a grant from the United States Public Health Service.

¹ Wheeler, A. H., and Nungester, W. J., *Science*, 1944, **100**, 523.

² Green, R. H., Rasmussen, A. F., Jr., and Smadel, J. E., *Pub. Health Rep., U. S. P. H. S.*, 1946, **61**, 1401.

³ Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, **85**, 623.

⁴ Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

⁵ Liebmann, A. J., Perlstein, D., and Snyder, G. A., *J. Bact.*, 1947, **54**, 63.

⁶ Rubin, B. A., and Giarman, N. J., *Yale J. Biol. and Med.*, 1947, **19**, 1017.

⁷ Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 99.

⁸ Burnet, F. M., *The Lancet*, 1948, **254**, 7.

⁹ Freund, J., *J. Immunol.*, 1931, **21**, 127.

Methods. The tannic acid employed was a commercial sample. The PR8 strain of influenza A virus was used exclusively. Allantoic fluid stored in a CO₂ chest was used as a source of virus. With minor differences, tests for inhibition were carried out as previously described.⁴

Experimental. Tannic acid was dissolved in water, usually in a concentration of 2 mg per cc, neutralized with sodium hydroxide, autoclaved and again adjusted to pH 7. 0.25 cc amounts of serial 2-fold dilutions of such solutions were mixed with 0.25 cc amounts of saline containing 8 to 16 hemagglutinating units of influenza A virus. After an interval of 5 to 15 minutes 0.25 cc of a 1% suspension of chicken erythrocytes was added to each tube. Tannic acid, in final concentration of from 5 to 20 µg per cc, inhibits hemagglutination.

Varying amounts of tannic acid were injected into the chorio-allantoic sacs of 10-day-old embryonated hens' eggs and after an interval of one-half hour 10 to 100 ID₅₀ of influenza A virus were inoculated by the same route. As a rule 0.5 cc containing one mg of tannic acid was injected. This amount of tannic acid inhibits the multiplication of virus to such an extent that the allantoic fluids from eggs so treated do not contain sufficient virus to produce hemagglutination. One mg of tannic acid injected into the allantoic sac at intervals up to 6 hours preceding the inoculation of virus as well as at intervals up to one hour following the inoculation of virus also causes inhibition of multiplication.

Allantoic fluid having an ID₅₀ titer of 10⁻⁷ was diluted 1-40 with saline and a portion of this was mixed with an equal volume of saline containing 0.2 mg of tannic acid per cc; as a control, another portion of the same

allantoic fluid was mixed with an equal volume of saline. After standing at 25°C for 15 minutes tests for infectivity were done by making serial 10-fold dilutions of each mixture and injecting aliquots of the various dilutions into the allantoic sacs of 10-day eggs. In such experiments tannic acid reduces the titer of virus by at least 3 logs.

Comment. The chemical effects of tannic acid on tissues are well known and have been summarized by Olitsky.¹⁰ However, there is little information available concerning its effect on viruses. Olitsky *et al.* showed^{10,11} that tannic acid instilled intranasally was able to prevent infection with the viruses of equine encephalomyelitis and poliomyelitis subsequently administered by the intranasal route. In this instance the action of tannic acid was said to be exerted on the nasal mucosa of the host rather than on the virus itself. The fact that tannic acid combines with proteins to form a material which shows a high degree of resistance to the destructive action of enzymes¹⁰ provides interesting ground for speculation as to the mechanism of its inhibitory action. In the experiments reported, however, it would appear that at least part of the inhibitory effect of tannic acid is dependent on a direct action upon the virus.

Summary. Tannic acid inhibits both hemagglutination and multiplication of influenza A virus. *In vitro*, the virus is inactivated by tannic acid.

It is a pleasure to acknowledge the technical assistance of Miss Ann Holloway.

¹⁰ Olitsky, P. K., and Cox, H. R., *Science*, 1934, **80**, 566.

¹¹ Olitsky, P. K., and Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 532.

Lack of Action of Influenza Virus upon Mucin of Human or Swine Origin.*

B. A. BRIODY† AND MARTIN HANIG. (Introduced by Thomas Francis, Jr.)

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Francis¹ reported that normal serum in high dilution could inhibit hemagglutination by heated influenza virus. Burnet, McCrea, and Anderson^{2,3} showed that (a) receptor destroying enzyme (RDE) of *Vibrio comma* and unheated influenza virus could destroy this inhibitor in normal serum, (b) a dilute solution of mucin derived from a pseudo-mucinous ovarian cyst and purified blood group substance A or O of human origin could inhibit hemagglutination by heated LEE influenza virus, and (c) RDE and unheated LEE virus could destroy this activity of mucin. The present report concerns the activity of influenza virus in the presence of purified preparations of human salivary mucin and gastric mucin of the hog.

The virus preparations were in the form of freshly harvested allantoic fluids of high titer derived from the infected chick embryo. The effect of various concentrations of human salivary mucin on 4 or 5 agglutinating doses of virus is recorded in Table I. The results show that as much as 2.5 mg/ml of mucin did not inhibit hemagglutination by heated or unheated influenza virus. No inhibition of hemagglutination was noted when gastric mucin of the hog was tested under the same conditions using swine influenza virus as well as the PR8 and LEE strains.

Since the salivary and gastric mucins did not inhibit hamagglutination, the technic

used by Burnet, McCrea, and Anderson³ could not be employed to determine whether or not influenza virus had any effect on mucin. However, an alternate technic utilizing the photoelectric colorimeter as a measure of turbidity was employed. In this type of experiment a small quantity of mucin in buffered saline was added to freshly harvested influenza virus. The results show that there was no decrease in the turbidity produced by mucin as a result of viral action (Table II). In addition the effect of the PR8 virus on both human salivary mucin and hog gastric mucin was studied to determine whether the virus could modify the conductivity or viscosity of mucin. In the conductivity test, there was no change in conductivity when 20 ml of PR8 virus with a final agglutinating titer of 1 to 40 acted on a solution of hog mucin or human salivary mucin containing 0.2 mg/ml of mucin in the presence of .0067M phosphate buffer at pH 7.4 at 25°C for 105 minutes and 204 minutes respectively. Using the Ostwald viscometer no alteration in viscosity of mucin was detectable in the presence of PR8 virus with a final agglutinating titer of 1 to 25 in .067M phosphate buffer at pH 7.4 when the virus acted on a 1% concentration of hog mucin and 0.25% concentration of human salivary mucin for 540 and 1017 minutes at 25°C respectively. These results are not surprising in view of the fact that Burnet, McCrea, and Anderson² have shown that the serological specificity of mucin is not destroyed by virus or by RDE. However, the clot or pellicle formed when a drop of the human salivary mucin or the gastric mucin was deposited in acid alcohol was dissolved by a drop of RDE thus indicating that these mucins were suitable substrates for RDE.

Two types of experiment were carried out to determine whether or not salivary or gastric mucin could inhibit the multiplication of in-

* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

† Fellow in the Medical Sciences of the National Research Council.

¹ Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

² Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

³ Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Austral. J. Exp. Biol.*, in press.

Methods. The tannic acid employed was a commercial sample. The PR8 strain of influenza A virus was used exclusively. Allantoic fluid stored in a CO₂ chest was used as a source of virus. With minor differences, tests for inhibition were carried out as previously described.⁴

Experimental. Tannic acid was dissolved in water, usually in a concentration of 2 mg per cc, neutralized with sodium hydroxide, autoclaved and again adjusted to pH 7. 0.25 cc amounts of serial 2-fold dilutions of such solutions were mixed with 0.25 cc amounts of saline containing 8 to 16 hemagglutinating units of influenza A virus. After an interval of 5 to 15 minutes 0.25 cc of a 1% suspension of chicken erythrocytes was added to each tube. Tannic acid, in final concentration of from 5 to 20 µg per cc, inhibits hemagglutination.

Varying amounts of tannic acid were injected into the chorio-allantoic sacs of 10-day-old embryonated hens' eggs and after an interval of one-half hour 10 to 100 ID₅₀ of influenza A virus were inoculated by the same route. As a rule 0.5 cc containing one mg of tannic acid was injected. This amount of tannic acid inhibits the multiplication of virus to such an extent that the allantoic fluids from eggs so treated do not contain sufficient virus to produce hemagglutination. One mg of tannic acid injected into the allantoic sac at intervals up to 6 hours preceding the inoculation of virus as well as at intervals up to one hour following the inoculation of virus also causes inhibition of multiplication.

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allantoic fluid was mixed with an equal volume of saline. After standing at 25°C for 15 minutes tests for infectivity were done by making serial 10-fold dilutions of each mixture and injecting aliquots of the various dilutions into the allantoic sacs of 10-day eggs. In such experiments tannic acid reduces the titer of virus by at least 3 logs.

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¹⁰ Olitsky, P. K., and Cox, H. R., *Science*, 1934, **80**, 566.

¹¹ Olitsky, P. K., and Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 532.

TABLE I.
Effect of Salivary Mucin on Hemagglutination by Viruses.

Virus	AD* of virus	Mucin mg/ml	Hemagglutination Dilution of mucin								Mucin control	
			4	8	16	32	64	128	256	512		1024
PRS	4	2.5	+	+	+	+	+	+	+	+	+	—
PRS heated 56°C/10	4	2.5	+	+	+	+	+	+	+	+	+	—
SW	5	10	+	+	+	+	+	+	+	+	+	—
SW " "	5	10	+	+	+	+	+	+	+	+	+	—
LEE	5	10	+	+	+	+	+	+	+	+	+	—
LEE " "	5	10	+	+	+	+	+	+	+	+	+	—

* AD—Agglutinating doses.

TABLE II.
Turbidimetric Readings of Virus-Mucin Mixtures.

Materials employed in test				Photoelectric colorimeter reading Time in hrs at 37°C				
Mucin mg/ml	LEE virus ml	NCAF ml	Saline ml	0	1.5	3.25	5	20
2.5	—	—	10	103	110	110	125	101
2.5	5	—	5	103	96	96	107	132
2.5	5	—	5	106	100	96	109	131
2.5	—	5	5	106	95	97	109	108
2.5	—	5	5	95	90	89	97	101
—	5	—	5	42	44	43	43	57

TABLE III.
Effect of Gastric Mucin on Infection of the Chick Embryo with LEE Virus

50% E.I.D.* of virus	Mucin mg/ml	RBC agglutination following inoculation		
		No. eggs	48 hrs after	72 hrs after
1	—	6	3/6	4/6
	12.5	6	1/6	2/6
10	—	6	4/6	4/6
	12.5	5	5/5	5/5
100	—	6	6/6	6/6
	12.5	6	2/6	3/6
1000	—	6	6/6	6/6
	12.5	5	5/5	5/5

* E.I.D.—Egg infective dose.

fluenza virus in the developing chick embryo. In Table III are recorded the results obtained when the dilution of virus used in the inoculum is prepared in a solution of mucin containing 12.5 mg/ml of mucin and the mixture held at room temperature for one hour prior to inoculation of 0.05 ml into the allantoic cavity. In the other type of experiment one ml of allantoic fluid was withdrawn from each egg to be inoculated and one ml of the mucin solution injected into the egg one hour

prior to inoculation with 0.05 ml of the virus dilution (Table IV). The results recorded in Table III and Table IV show that there is no inhibition of influenza virus multiplication when as much as 25 mg of mucin is introduced into the allantoic cavity prior to inoculation with ten 50% infective doses.

Comment. The evidence presented by Burnet and his colleagues^{2,3,4} strongly sug-

⁴ Burnet, F. M., *Lancet*, 1948, 1, 7.

TABLE IV.
Effect of Salivary Mucin on Infection with LEE and PR8 Viruses.

50% E.I.D.* of virus	Mucin mg	Time mucin given in relation to virus inoculation	No. eggs	RBC agglutination 48 hrs after inoculation
100 LEE	5	1 hr before	5	5/5
100 "	—	—	6	6/6
10 PR8	25	1 " "	5	4/5
10 "	—	—	6	6/6

* E.I.D.—Egg infective dose.

gested that influenza virus has a direct enzymic action on mucin in the form of purified blood group substances. Burnet⁵ thinks that the chief significance of this finding is that it provides a partial basis for the specific localization of infection by viruses of the mumps-influenza group to glands and mucous surfaces. Our failure to secure any evidence of viral action on human salivary mucin and hog gastric mucin indicates that the chemical composition and/or the molecular configurations of mucins derived from different sources are not identical. Supporting evidence for this viewpoint is contained in a recent report which shows that there are wide variations in the physical characteristics including the isoelectric point of mucin obtained

from various levels of the gastro-intestinal tract of the hog.⁶ The alteration of the mucinous blood group substances does not result in any alteration of the serological specificity of mucin² which again suggests that the change might involve only a slight molecular rearrangement.

Summary. Human salivary mucin and hog gastric mucin do not inhibit the hemagglutination induced by the PR8, LEE, and Swine strains of influenza virus and these mucins do not inhibit the multiplication of PR8 or LEE in the developing chick embryo. There was no turbidimetric or viscosimetric evidence of viral action on these mucins. Influenza virus was also unable to modify the conductivity of the human salivary mucin or the hog gastric mucin.

⁵ Burnet, F. M., *Austral. J. Science*, 1947, 10, 21.

⁶ Domini, G., *Arch. fistol.*, 1941, 41, 36.

16349

Effects of Ether and Nembutal Anesthesia upon Blood Concentration of the Rat.

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The effects of ether and nembutal anesthesia on the blood concentration have been reported by several investigators. Anesthesia by nembutal results in hemodilution in cats¹ and in dogs.^{2,3} Anesthesia by ether has been found

to produce hemoconcentration in dogs^{4,7} and

¹ Hamlin, H., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, 1939, 125, 713.

² Hausner, E., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, 1938, 121, 357.

³ Hahn, P. F., Bale, W. F., and Bonner, J. F., Jr., *Am. J. Physiol.*, 1943, 138, 415.

⁴ Barbour, H. G., and Bourns, W., *Am. J. Physiol.*, 1924, 67, 399.

⁵ McAllister, F. F., *Am. J. Physiol.*, 1937, 119, 363; 1938, 124, 391.

⁶ Bollman, J. L., Svirbely, J. L., and Mann, F. C., *Surgery*, 1938, 4, 881.

⁷ Searls, P. W., *J. A. M. A.*, 1939, 113, 906.

a similar but less marked effect is believed to appear in man.^{8,9} However, in etherized cats¹⁰ and rabbits¹¹ no such blood concentration occurs.

It is clear that a species difference exists in the response of the above animals to ether, and with further investigation a difference may appear among other experimental animals. In this respect the laboratory rat has been strangely neglected; no study of the effect of ether or nembutal on the blood concentration of the rat has been reported in the literature.

The problem introduced is important on two counts: (1) the correct interpretation of blood data obtained from anesthetized animals requires a knowledge of the changes produced by the anesthesia employed; (2) there is danger in applying information gained from one species to experiments involving another.

Methods. Male albino rats, weighing about 200 g, were studied in 2 groups of 20 rats each, for the effect of anesthesia on the blood concentration. The effects of ether and nembutal were determined on separate groups. Ether was administered by the respiratory route until the animal was prostrate and all reflexes absent. Nembutal was given by intraperitoneal injection in the amount of 0.2 ml of a 10% solution. The rats were placed in a restraining box and blood samples were taken before and immediately after anesthesia by clipping the tail and using the free-flowing blood. Additional samples were obtained following anesthesia by the use of cardiac puncture. Determinations were made on the red cell count and on the specific gravity of the blood. The cell count was determined by employing standard clinical dilution pipettes, diluting fluids, and hemocytometers. The specific gravity was determined by the copper sulfate method of Phillips *et al.*¹²

Results. It is clear from the averages in

TABLE I.

Effect of Anesthesia by Nembutal and by Ether on the Erythrocyte Number of the Blood of the Albino Rat. 20 Rats for Each Determination.

Treatment and source of blood	Red blood cells	
	Mean* × 1000	Range × 1000
No anesthesia; tail	8,530 ± 130	(7,220-9,780)
Nembutal; tail	7,802 ± 109	(6,430-8,650)
" heart	7,376 ± 136	(6,330-8,180)
No anesthesia; tail	8,368 ± 200	(7,440-9,384)
Ether; tail	7,665 ± 210	(7,106-8,926)
" heart	6,965 ± 88	(6,240-7,910)

TABLE II.

Effect of Anesthesia by Nembutal and by Ether on the Specific Gravity of the Blood of the Albino Rat. 20 Rats for Each Determination.

Treatment and source of blood	Specific gravity	
	Mean*	Range
No anesthesia; tail	1.0603 ± .0002	(1.059-1.062)
Nembutal; tail	1.0549 ± .0003	(1.053-1.058)
" heart	1.0529 ± .0003	(1.051-1.055)
No anesthesia; tail	1.0584 ± .0003	(1.058-1.061)
Ether; tail	1.0548 ± .0002	(1.053-1.057)
" heart	1.0521 ± .0001	(1.050-1.055)

* Including standard deviation of the mean.

Tables I and II that both ether and nembutal produced a hemodilution as expressed in the reduced red cell numbers and specific gravity. These differences have been shown to be significant by application of the Fishers' *t* test. The greater dilution of the blood drawn from the heart as compared to that taken from the tail is due to the longer time over which the anesthesia was allowed to act. This is supported by the fact that in a separate study red cell counts made on blood samples taken simultaneously from the tail and heart of a group of ten anesthetized rats were nearly identical, the means being 7,651,000 and 7,513,000 respectively. Similar counts on a group of ten unanesthetized rats also showed the blood from these two sources to be nearly identical, the numbers being 8,967,000 and 8,790,000.

⁸ Gibson, J. G., 2nd, and Branch, C. D., *Surg., Gyn. and Obstet.*, 1937, **65**, 741.

⁹ Ragan, C., Ferrebee, J. W., and Fish, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 712.

¹⁰ Conley, C. L., *Am. J. Physiol.*, 1941, **132**, 796.

¹¹ Barbour, H. G., *Anesthesiology*, 1940, **1**, 121.

¹² Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, Josiah Macy, Jr., Foundation, New York, Feb., 1945.

Summary. Anesthesia in the rat by means of nembutal or ether produces a hemodilution, as shown by both a decrease in the red cell numbers and in the specific gravity of the blood.

16350

Possible Eczematous Cross-Hypersensitivity Between Paraphenylenediamine and Azo-Dyes Certified for Use in Foods, Drugs and Cosmetics.*

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In recent reports Dobkevitch and Baer^{1,2} have analyzed in a number of patients the cross-sensitization between paraphenylenediamine and certain azo-dyes used in the manufacture of nylon stockings. It was found that this cross-sensitization represented a new example of the complex of hypersensitivity to compounds of quinone structure, as described by Mayer.^{3,4} The present study was undertaken to ascertain, in subjects with allergic eczematous contact-type sensitization to paraphenylenediamine, whether this cross-sensitization extends not only to those azo-dyes used in the dyeing of various goods or as therapeutics for dermatological purposes, but also to those azo-dyes which are commonly used in the United States as dyes, for foods, drugs and cosmetics.

The use of synthetic dyes in foods, drugs and cosmetics is regulated under authority of the Federal Food, Drug and Cosmetic Act of 1938 and no coal tar colors other than those listed and described in these regulations may be used in foods, drugs and cosmetics. Among the 116 dyes listed, 18 are accepted

for use in foods, 10 of which are azo-dyes. These 10 dyes are "straight" colors, free from all impurities other than a specified maximal allowance (5%) for chlorides and sulfates of sodium; for certain aromatic amines, as o-toluidine, 0.05%, permitted in FD&C Orange No. 1; or xylydine, 0.1%, in FD&C Red No. 32; for certain aromatic hydrocarbons, as betanaphthol, 0.05% in the above-mentioned dyes; and permitting only traces of metals such as arsenic (0.00014%) and lead (0.001%).

All colors used in foods have been examined by the Food and Drug Administration and found to be harmless in the toxicologic sense. The present regulations⁵ do not set any standards for ascertaining the eczematogenic potential of dyes to be used in foods, drugs and cosmetics, although all of the certified colors have been tested for sensitizing capacity on the skin of groups of guinea pigs (Calvery⁶).

Experimental. Twenty-five subjects with known allergic eczematous contact-type hypersensitivity to paraphenylenediamine and 21 control subjects with no hypersensitivity to this compound but most of them with hypersensitivity to other non-related substances were given patch tests with the following substances:

* With the technical assistance of Mrs. Dorothy Furman.

† Chief Microbiologist, Ciba Pharmaceutical Products, Inc., Summit, N.J.

¹ Dobkevitch, S., and Baer, R. L., *J. Invest. Dermat.*, 1947, **8**, 419.

² Dobkevitch, S., and Baer, R. L., *J. Invest. Dermat.*, 1947, **9**, 203.

³ Mayer, R. L., *Arch. f. Dermat. u. Syph.*, 1928, **150**, 312.

⁴ Mayer, R. L., *J. Invest. Dermat.*, 1948, **10**, 389.

⁵ Federal Security Agency, Food and Drug Administration: Coal-Tar Color Regulations, September, 1940.

⁶ Calvery, H. O., *Am. J. Pharm.*, 1942, **114**, No. 9.

TABLE I.
Chemical Terminology of Dyes.

No.	Designation	Composition
1	FD&C Red No. 1	Disodium salt of 1-pseudoecumylazo-2-naphthol-3,6-disulfonic acid
2	" " " 2	Trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid
3	" " " 4	Disodium salt of 2-(5-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid
4	" " " 32	1-xylylazo-2-naphthol
5	" Yellow No. 3	1-phenylazo-2-naphthylamine
6	" " " 4	1-o-tolylazo-2-naphthylamine
7	" " " 5	Trisodium salt of 3-carboxy-5-hydroxy-1-p-sulfophenyl-4-p-sulfophenyl-azopyrazole
8	" " " 6	Disodium salt of 1-p-sulfophenylazo-2-naphthol-6-sulfonic acid
9	" Orange No. 1	Monosodium salt of 4-p-sulfophenylazo-1-naphthol
10	" " " 2	1-o-tolylazo-2-naphthol

(a) Ten azo-dyes certified for use in foods, drugs and cosmetics according to the Federal Food, Drug and Cosmetic Act, (b) paraphenylenediamine, and (c) a sample of nylon stocking material dyed, among others, with azo-dyes.

The procedure adopted for patch testing with these substances was that described by Dobkevitch and Baer.² All dyes were used in 2% concentrations in white petrolatum. In many instances the reading of the tests was difficult since the dyes, particularly the red, conferred upon the skin a color sometimes indistinguishable from simple erythema.

The chemical terminology of these dyes is given in Table I.

Results. The results of the patch tests are summarized in Tables II and III.

In the group of subjects who were hypersensitive to paraphenylenediamine (Table II), 4 of the 10 azo-dyes tested elicited strongly positive reactions (2+ to 3+) in several patients: FD&C Red No. 32, FD&C Yellow No. 3, No. 4 and No. 6. Occasional reactions were produced by FD&C Red No. 2 and FD&C Orange Nos. 1 and 2.

In the group of control subjects, not sensitive to paraphenylenediamine, (Table III), 3 of the 10 azo-dyes elicited marked positive (2+) reactions: FD&C Red No. 32, FD&C Yellow No. 3 and FD&C Orange No. 2. Slightly positive reactions (+) and 1+ were occasionally produced by the dyes FD&C Red Nos. 1 and 4 and FD&C Yellow No. 4.

The 3 dyes which produce the strongest primary reactions in the group of patients not sensitive to paraphenylenediamine are among the 4 dyes which produce the strongest

reactions in the group of patients sensitive to paraphenylenediamine. Our inference is that in the concentration used, these dyes are primary irritants.

However, an analysis of the results reveals certain differences between the reactivities of the two groups of patients towards these dyes. Generally, the reactions produced in the non-paraphenylenediamine-hypersensitive control group are not only fewer in number, but they are definitely weaker, 10 1+ to 2+ and 4 2+ reactions being elicited in 210 tests. In the group of the paraphenylenediamine sensitive patients there were 13 1+ to 2+, 5 2+ and 14 stronger positive reactions in 250 tests. Dye FD&C Yellow No. 3, for instance, gave the following results: In the control group, 3 patients had 2+ reactions; all the others showed a weaker reaction or were negative (9 out of 21). In the group of patients sensitive to paraphenylenediamine, 8 patients had 2+ or stronger reactions and only 6 out of 25 were negative. Similar results were obtained with the other dyes.

Contrary to the 3 aforementioned dyes which had produced many positive reactions in both groups, FD&C Yellow No. 6 is not a primary irritant, since it produced one 1+ reaction in only one of the controls but produced 8 reactions, 4 of which were 2+ to 3+, among the patients sensitive to paraphenylenediamine.

Discussion. The tests reported in Table III on patients not sensitive to paraphenylenediamine show that the dyes FD&C Yellow No. 3, Orange No. 2, Red No. 32 and to a lesser extent, Yellow No. 3 must be considered as primary irritants in the concentra-

TABLE II.
Patch Tests in Subjects Hypersensitive to Paraphenylenediamine.

Patient	FD&G Red No. 1	FD&G Red No. 2	FD&G Red No. 4	FD&G Red No. 32	FD&G Yel. No. 3	FD&G Yel. No. 4	FD&G Yel. No. 5	FD&G Yel. No. 6	FD&G Or. No. 1	FD&G Or. No. 2	Para- phenylene- diamine	Nylon Stock- ings
P21768	0	0	0	+	+	(+)	0	+	+	+	+	+
P26922	0	0	0	(+)	+	(+)	0	+	0	+	+	+
C 17908	0	0	(+)	+	+	+	0	+	0	(+)	+	+
P22960	?	?	?	+	+	?	0	+	0	0	+	+
P27324	0	0	0	+	+	0	0	+	0	0	+	+
P31914	0	0	0	+	+	0	0	+	0	0	+	+
P34347	0	+	0	+	+	0	0	+	(+)	0	+	+
P33237	0	0	0	+	0	+	+	+	0	+	+	+
P37037	0	0	0	+	+	+	+	+	0	+	+	+
P32987	0	0	0	0	+	+	+	0	0	+	+	+
P39973	0	0	0	+	+	0	0	0	0	+	+	+
P33388	0	0	0	0	0	0	0	0	0	+	+	+
P31513	0	0	0	+	+	0	0	0	0	+	+	+
P39396	0	0	0	+	(+)	0	0	0	0	+	+	+
P35345	0	0	0	+	+	0	0	0	+	+	+	+
P29970	0	0	0	+	(+)	0	0	0	0	0	+	+
P26266	0	0	0	0	+	0	0	0	0	+	+	+
P27324	0	0	0	(+)	+	+	0	0	0	+	+	+
D10124	0	0	0	(+)	+	?	?	+	0	?	+	+
P44406	0	0	0	+	(+)	(+)	0	+	0	+	+	+
P22965	0	0	0	?	+	(+)	0	0	0	+	+	+
P4385	0	0	0	+	(+)	+	0	0	0	+	+	+
P44384	0	?	?	+	+	+	?	?	?	+	+	+
P44400	0	0	?	+	+	0	0	0	0	+	+	+
P44932	0	0	0	+	+	0	0	0	0	+	+	+

TABLE III.
Patch Tests in Subjects Not Hypersensitive to Paraphenylenediamine (Control Group).

Patient	FD&C Red No. 1	FD&C Red No. 2	FD&C Red No. 4	FD&C Red No. 32	FD&C Yel. No. 3	FD&C Yel. No. 4	FD&C Yel. No. 5	FD&C Yel. No. 6	FD&C Or. No. 1	FD&C Or. No. 2	Para- phenylene- diamine	Nylon Stock- ings
F31491	0	0	0	0	0	0	0	0	0	0	0	0
F31702	0	0	0	0	0	0	0	0	0	+	0	0
F19511	(+)	0	+	+	+	(+)	0	+	0	+	?	0
F40196	+	0	0	0	0	0	0	0	0	+	0	0
C46823	0	0	0	0	+	(+)	0	0	0	+	0	0
F40521	(+)	0	0	(+)	0	(+)	0	0	0	+	0	0
F41148	0	0	0	0	+	(+)	0	0	0	+	0	0
F41871	0	0	0	+	+	?	0	0	0	+	0	0
F42607	0	0	0	0	0	0	0	0	(+)	0	0	0
A54758	0	0	0	0	?	0	0	0	0	?	0	0
F44451	0	0	(+)	+	+	+	0	0	0	+	0	0
F44021	0	0	0	(+)	+	+	0	0	0	+	0	0
F44304	0	0	0	?	0	0	0	0	0	(+)	0	0
F45131	?	0	0	+	0	0	0	0	0	+	(+)	0
F44868	0	0	(+)	+	+	0	0	0	0	+	0	0
F15719	0	0	+	(+)	+	0	0	0	0	+	0	0
F44462	0	0	0	+	+	0	0	0	0	+	0	0
F38568	0	0	0	+	+	+	0	0	0	+	0	0
F44014	0	0	0	+	+	+	0	0	0	+	0	0
F45131	0	0	0	?	?	0	0	0	0	+	0	0
F45521	0	0	0	+	+	?	0	0	0	+	0	0

tion chosen for the tests. One could therefore question whether positive skin reactions elicited with these dyes in the group of patients sensitive to paraphenylenediamine are expressions of an allergic cross-sensitivity to compounds of quinone structure and not merely coincidental reactivities produced by primary irritants.

Two facts, however, favor the supposition that these reactions are etiologically associated with the sensitivity to paraphenylenediamine and not merely coincidental. (1) Both the number and the strength of the skin reactions produced by these dyes are definitely greater in the group of patients sensitive to paraphenylenediamine than in the control group consisting of patients not sensitive to paraphenylenediamine, but sensitive to other allergens. These dyes elicited 14 reactions stronger than 2+ in the patients hypersensitive to paraphenylenediamine; and no reactions stronger than 2+ in the control group. (2) Although dye FD&C Yellow No. 6 was not a primary irritant in the concentration employed, it produced skin reactions in 8 of the patients sensitive to paraphenylenediamine. In 5 of these cases the strength of the reactions closely paralleled their reactions to paraphenylenediamine. We therefore believe that among the positive reactions produced by the various dyes in paraphenylenediamine sensitive patients, there are several elicited by a specific skin sensitivity to compounds of quinone structure.

The results obtained suggest that the eczematous hypersensitivity to paraphenylenediamine not only crosses over to some azo-dyes which are used for dyeing leather, fabrics and other goods, but also to certain food dyes, such as FD&C Yellow No. 6 which is commonly used for coloring beverages, FD&C Red No. 32 used in external coloring of oranges and FD&C Yellow Nos. 3 and 4, both used in oleomargarine and butter.

It is by no means surprising that certain azo-dyes employed in coloring food are capable of producing cross-reactions in patients sensitive to paraphenylenediamine. From the chemical standpoint these food colors are not fundamentally different from those azo-dyes

which are used for technical or pharmaceutical purposes, their only distinctive property being their specific purpose and the fact that they are more highly purified.

For obvious reasons these azo-dyes are less conspicuous as general allergens than the related azo-compounds which are used for other purposes. The production of sensitizations and the elicitation of the reactions of hypersensitivity depend to a great extent not only on the intrinsic sensitizing power of the antigen, but also on the concentration and on the intensity with which the material comes in contact with the susceptible cells.

There are indeed quantitative differences in the amounts of dye usually absorbed by the body from foods and those coming in contact directly with the skin from dyed goods. The quantities of azo-dyes ingested with each meal and then reach the cells of the skin must be much smaller than those which reach the skin from dyed fabrics, furs or from medicinal preparations.

As is often the case under similar circumstances, the question arises whether the positive patch tests elicited by the food dyes in patients sensitive to paraphenylenediamine are of practical importance. It remains to be shown that patients presenting these positive reactions will react with clinical symptoms to the ingestion of foods colored with the incriminated dyes.

We have had one patient, sensitive to paraphenylenediamine, eat within 48 hours, one pound of oleomargarine dyed with dye FD&C Yellow No. 3 but the ingestion was not followed by any clinical manifestations of hypersensitivity. However, further trials of this type and particularly chronic and repeated exposures by ingestion are necessary; only then will it be known whether the amount of azo-dye ingested with food is sufficient to elicit reactions in individuals sensitized to compounds of quinone structure or will cause sensitizations in normal individuals. This problem is worthy of serious consideration since a very large portion of the population of the United States is continually exposed to many of the certified azo-dyes by contact, ingestion and probably also inhalation and

the possibility of their reacting to these substances may constitute a serious problem.

Certain other problems are intimately related to this question. It is known, for example, that a number of cases of dermatitis from paraphenylenediamine and related compounds used in hair and fur dyes, photo developers, leather dyes, nylon stockings, etc. occasionally show flare-ups which cannot be explained on the basis of a new exposure to the causative agent. Our results suggest the possibility that the ingestion of certified azo-dyes, which have been shown to produce reactions in patients hypersensitive to paraphenylenediamine, may be responsible for these recurrences. Furthermore, it may be possible that certain eczematous eruptions now attributed to various foods themselves are actually due to the azo-dyes contained in these foods. A study of the influence of continuous exposure to these dyes on the causation and persistence of non-eczematous and possibly non-cutaneous allergies as, for example, asthma due to paraphenylenediamine and related compounds, would prove of great interest.

In evaluating the results shown in Table II it seems at first glance inexplicable that not all aminated azo-dyes used in foods, cosmetics and drugs produce irritations. While certain of the food dyes listed in Table II

are seemingly harmless, even for patients highly sensitive to paraphenylenediamine, other dyes of closely related constitution elicit strong skin reactions in a high percentage of cases. The data gathered to date in various studies indicate that the presence or absence of a response to a given azo-dye in a case of cross-sensitization to compounds of quinone structure depends essentially upon the chemical structure of the dyes. Indeed, the capacity of an azo-dye to produce skin reactions in patients sensitive to paraphenylenediamine seems to be dependent in most cases upon the ease of its transformation into compounds of quinone structure and upon the ability of the quinone compound thus formed to couple with certain body constituents. We believe that only those azo-dyes capable of undergoing these changes have sensitizing power and elicit reactions in an appreciable number of cases.¹

Conclusions. Skin tests with certain certified food dyes in patients sensitive to paraphenylenediamine suggest that eczematous hypersensitivity to paraphenylenediamine may not only cross over to azo-dyes used for the dyeing of leather and fabrics, or in ointments, but also to certain azo-dyes certified by the Food, Drug and Cosmetic Act of 1938 for use in foods, drugs and cosmetics. The implications of these findings are discussed.

16351

Effect of Pneumonectomy and of Lung Extract on Experimental Renal Hypertension.*

B. Z. KLATCH AND G. E. WAKERLIN.

From the Department of Physiology, University of Illinois College of Medicine.

Katz and Steinitz¹ have demonstrated in dogs that pulmonary arterial pressure is not altered in experimental renal hypertension. This may be explained by the inability of

the pulmonary arterioles to respond adequately to the renal pressor mechanism, inasmuch as Brenner² found that the walls of these vessels average 5.7% of the external

* Aided by grants from the John and Mary R. Markle Foundation, the United States Navy, and the Graduate School Research Board of the University of Illinois.

¹ Katz, L. M., and Steinitz, F. S., *Am. J. Physiol.*, 1940, **128**, 433.

² Brenner, O., *Arch. Int. Med.*, 1935, **56**, 211, 457, 724, 976, 1189.

diameter, compared with 36% for the systemic arterioles. Certain experimental data, however, demonstrate that the pulmonary arterioles do respond to pressor agents. Friedberg *et al.*³ showed that the intravenous injection of angiotonin produced a rise in pressure in the pulmonary artery of the dog occurring almost simultaneously with the systemic rise. With renin, the pulmonary pressure rise lagged behind the systemic. Binet and Bergeton⁴ observed a vasoconstrictor action of renin on the vessels of the isolated dog lung, and found that renin is inactivated by passage through a heart-lung preparation. Braun-Menendez *et al.*,⁵ however, observed no appreciable diminution in renin activity after 2 hours of perfusion through a heart-lung preparation. These findings suggest either that renin and angiotonin are not involved in hypertension of renal origin, or that in chronic renal hypertension the pressor mechanism may operate differently in the pulmonary as compared with the systemic circulation.

Design of experiments. The pulmonary normotension in experimental renal hypertension may be due to the elaboration by the lung of an antihypertensive substance or to the lung's inactivation of a pressor agent produced by the kidney. This may occur to an extent sufficient to prevent the development of pulmonary hypertension, yet not to inhibit the production of an elevated pressure in the systemic circuit.

To test for an antihypertensive agent in lung tissue, 2 dogs were injected with lung extract for 2 months, then were subjected to a unilateral renal artery constriction and a contralateral nephrectomy. Injections were continued throughout the operation period and for 2 months following. Good evidence exists that prophylaxis can be obtained by a similar injection procedure with certain renal

extracts.⁶ The technique used in constricting the renal arteries has been found to result in the development of hypertension in a high percentage of control dogs.

To investigate the effect of a reduction in lung substance, pneumonectomy was performed on 4 dogs with moderate renal hypertension. Two of these dogs were later subjected to contralateral lobectomy. It was felt that any possible pressor inactivating effect of the lungs might thereby be reduced to a degree sufficient to permit the hypertensive substance elaborated by the kidney to raise the systemic pressure to a still higher level. Two normotensive dogs were pneumonectomized as controls. Unilateral renal artery constriction and contralateral nephrectomy were later done on these controls.

Procedures. Arterial pressures were obtained on the animals by direct femoral puncture at least twice a week.⁷ The renal arteries were clamped according to our modification of the Goldblatt technique⁸ which produces hypertension in practically 100% of dogs. The pneumonectomies and lobectomies were performed according to the technique of Joannides.⁹

The lung extract was prepared from dog lungs removed immediately after death. The lungs were frozen and ground in a meat chopper. The tissue was then defatted over 48 hours by 2 changes of cold acetone and for another 24 hours by cold ether. It was then dried under vacuum at room temperature in a drying column and finely divided in a coffee grinder. Fresh extracts of this lung powder were made with a .5% NaHCO₃ in physiological salt solution. One cc of the final extract was equivalent to one gram of fresh tissue. Streptomycin and penicillin were added as preservative. The animals were injected intramuscularly 6 days a week with a dose of 1.5 cc per kg of body weight.

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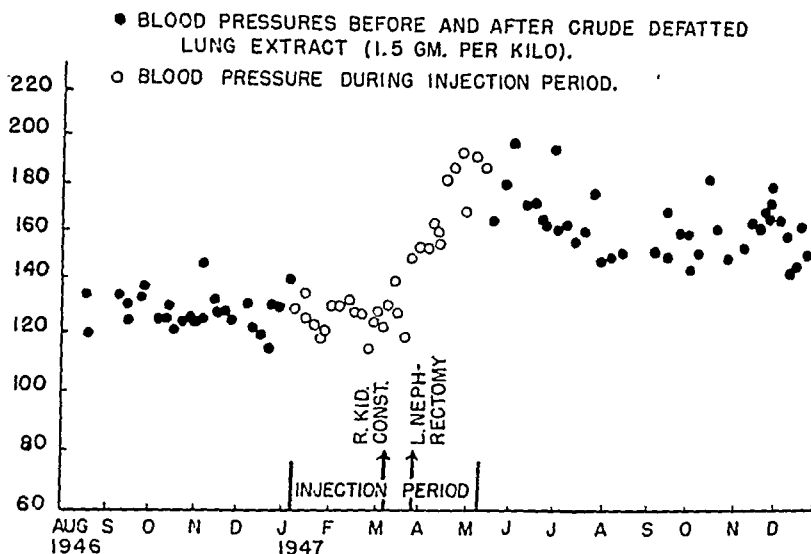


FIG. 1.

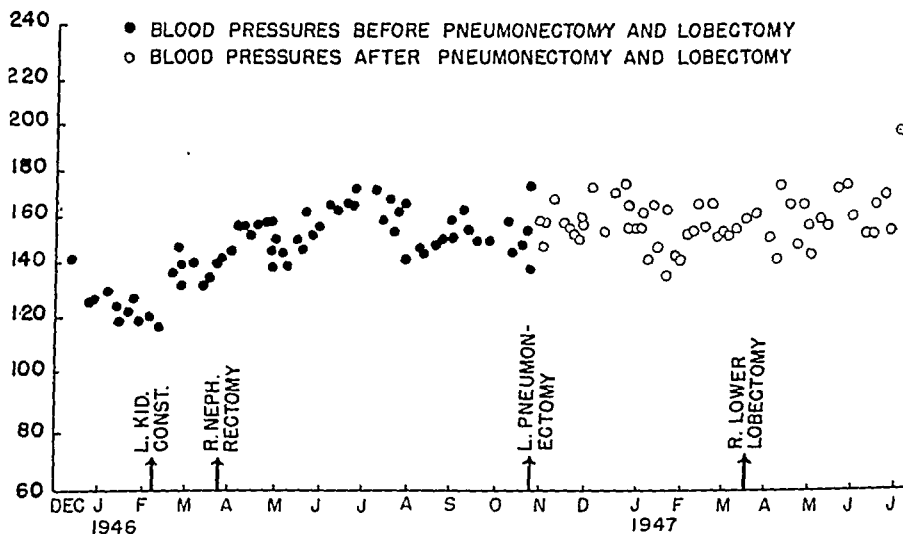


FIG. 2.

Results. 1. The lung extract failed to prevent the development of hypertension in the 2 injected dogs following constriction of the renal artery and contralateral nephrectomy (Fig. 1). All ten of the control dogs developed hypertension, 2 of them dying of malignant hypertension. 2. Pneumonectomies performed on 2 of the 4 moderately hypertensive dogs did not alter in any way their pressure over a period of 4 months. 3. Pneumonectomy and

contralateral lobectomy on the other 2 hypertensive dogs likewise did not alter their pressure over a period of 6 months (Fig. 2). 4. The normotensions of 2 dogs were not influenced by pneumonectomy, nor did the absence of a lung alter the hypertension produced by subsequent renal artery clamping and contralateral nephrectomy.

Discussion. The results demonstrate that the lung extract used had no prophylactic

antihypertensive effect. Moreover, a preliminary experiment with the dog lung extract in rats likewise indicated no prophylaxis against experimental renal hypertension.¹⁰ However, antihypertensive substances not obtained by this method of extraction may be present in lung.

A reduction in the amount of functioning lung tissue was shown to be without effect on the systemic blood pressure of moderately hypertensive animals. No regeneration of lung tissue was found in these dogs on sacrifice, and it seems likely that sufficient tissue was removed to demonstrate some effect if the lung plays an active antipressor role in experimental renal hypertension.

Summary. 1. Daily intramuscular injections of lung extract for two months preceding and following clamping of the renal artery and

contralateral nephrectomy failed to prevent the development of hypertension in two dogs. 2. Pneumonectomy performed on 2 renal hypertensive dogs and pneumonectomy plus contralateral lobectomy on 2 other hypertensive animals did not alter their pressure levels. 3. The pressures of 2 normotensive dogs were not influenced by pneumonectomy, nor were the hypertensions subsequently produced by renal artery constriction and contralateral nephrectomy. 4. The results of these experiments suggest, but do not prove, that the lungs do not elaborate an antihypertensive substance and do not inactivate the pressor agent of experimental renal hypertension. 5. Further work is necessary to explain the pulmonary normotension of renal hypertensive dogs.

We are grateful to Dr. M. Joannides for performing the pneumonectomies and lobectomies.

¹⁰ Klatch, B. Z., unpublished data.

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Relationship Between the pH of the Duodenal Content and Pancreatic Secretion.*

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Bayliss and Starling¹ demonstrated the mechanism whereby acid introduced into the intestine provoked a flow of pancreatic secretion. With increasing interest in the role of the endocrine glands in the regulation of bodily function it has been widely assumed that the secretin mechanism largely determined the volume of pancreatic secretion.² Farrell and Ivy,³ who feel the secretin mech-

anism is the most important, found that a denervated pancreas failed to secrete as much as expected. McClure,⁴ on the basis of his determination of enzyme in the duodenal content, stated that "stimulation of the external enzymic function of the pancreas is dependent on some factor or factors unrelated to acidity of intestinal contents." Thomas

* The work was supported in part by a grant from the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

¹ Bayliss, W. M., and Starling, E. H., *Am. J. Physiol.*, 1902, **28**, 325.

² *Howell's Text Book of Physiology*, 15th Edition, 1946, p. 1064, W. B. Saunders Co., Philadelphia; *Physiology in Health and Disease*, C. S. Wiggers, 4th Edition, 1947, p. 809, Lea and Febiger, Philadelphia.

³ Farrell and Ivy, *Am. J. Physiol.*, 1926, **78**, 325.

⁴ McClure, C. W., *Functional Activities of the Pancreas and Liver*, Medical Authors Publishing Co., 1937.

⁵ Thomas, J. E., and Crider, J. O., *Am. J. Physiol.*, 1940, **131**, 349.

and Crider⁵ found that acid, as a stimulant for pancreatic secretion was effective when the pH was about 4; however, a copious flow of pancreatic juice was produced only when the pH was reduced to 3 or less. The latter investigators also⁶ found that, in the dog, after a meat meal, the pH of the duodenal content generally hovered around 4, at which level, a threshold response only would be expected.

Since pancreatic juice with a pH of about 8 is secreted into a vulnerable part of the duodenum, and is an excellent buffer, it is generally believed that this secretion plays a decisive role in the neutralization of acid chyme.⁷ Hoerner⁸ showed that even when the pancreatic juice was excluded from the duodenum, a significant degree of neutralization of the acid gastric contents occurred. On the other hand, DeBakey⁹ felt that the pancreatic juice played a lesser role than did bile in the prevention of ulcer in the intestinal tract.

In the course of study undertaken to determine the normal secretion of the pancreas after the ingestion of various foodstuffs, observations were made which yielded information pertaining to both of these problems.

Methods. Healthy adult dogs were provided with gastric and duodenal tubulated fistulae as has been described elsewhere.¹⁰ The accessory pancreatic duct was doubly ligated and severed; the duodenal fistula was placed opposite the ostium of the main pancreatic duct. (Fig. 1). After complete recovery from the above operation, the animal was placed in a standing position on a table by means of a comfortable muslin harness. A glass cannula was introduced into the main pancreatic duct; two tubes were placed into

the stomach and then through the pylorus into the duodenum, the shorter tube being so placed that its end was in the region of the orifice of the main pancreatic duct while the end of the longer tube was placed 3 inches beyond this point. The gastric fistula was stoppered and the duodenal fistula was covered with a rubber dam which was pierced to permit the small pancreatic cannula to protrude to the outside.

Observations were made on 3 dogs so prepared. The pancreatic juice was collected and the volume measured every 10 minutes. In one series of experiments, each 10-minute specimen was introduced into the duodenum through the proximal tube; in a second group the pancreatic juice was excluded from the intestine during the period of observation. Samples of duodenal content were withdrawn from the longer tube at intervals and the pH determined electrometrically, using a glass electrode. These samples were then promptly returned to the duodenum. Preliminary observations were made in the fasting state for one hour, after which the animal was fed small amounts of different foodstuffs and observations were continued for five hours. In a few experiments large meals were fed, and observations continued for a longer period of time.

Results. A comparison of the pH determinations of the duodenal contents made when the pancreatic juice was excluded from the intestine with the observations made when it was reintroduced, reveals that although, in general, the pH is lower when the juice is not present, the acidity did not exceed that observed when the juice was replaced into the duodenum. The difference between the 2 groups of experiments is evident when individual foodstuffs are considered separately. There is a tendency for the pH to be higher when the pancreatic juice is present.

Fig. 2 presents this data *in toto*. The ordinates represent the pH determined after the ingestion of food, the abscissae denote the volume of pancreatic juice secreted in the ensuing 10-minute period. Entirely similar results were obtained when the volume of pancreatic juice secreted during the same ten-

⁶ Thomas, J. E., *Am. J. Dig. Dis.*, 1940, **7**, 195.

⁷ Mann, F. C., and Bollman, J. L., *Am. J. Dig. Dis.*, 1935, **2**, 284; Hoerner, M. T., *Am. J. Dig. Dis.*, 1935, **2**, 300.

⁸ Hoerner, M. T., *Am. J. Dig. Dis.*, 1935, **2**, 288.

⁹ DeBakey, M. E., *Arch. Surg.*, 1937, **34**, 230.

¹⁰ Scott, V. Brown, Collingnon, W. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 248; Hart, Wm. M., and Thomas, J. E., *Gastro-enterology*, 1945, **4**, 409.

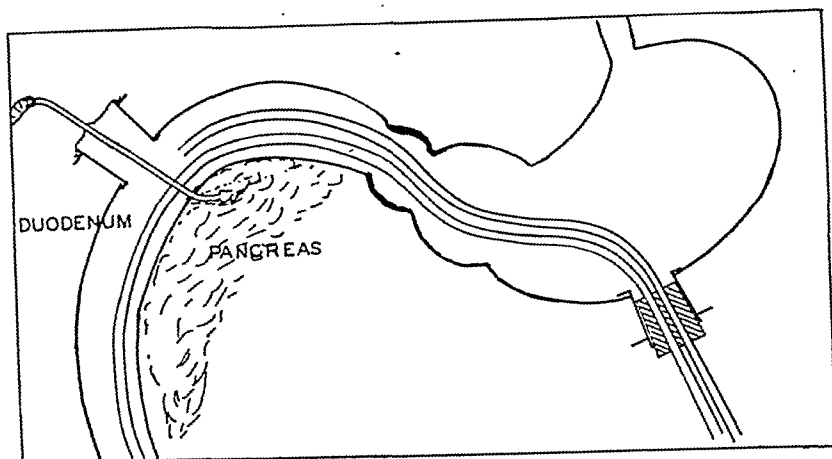


FIG. 1.

Diagram showing relationships of the gastric and duodenal fistulas and the pancreatic cannula; also, the position of the two tubes introduced into the duodenum.

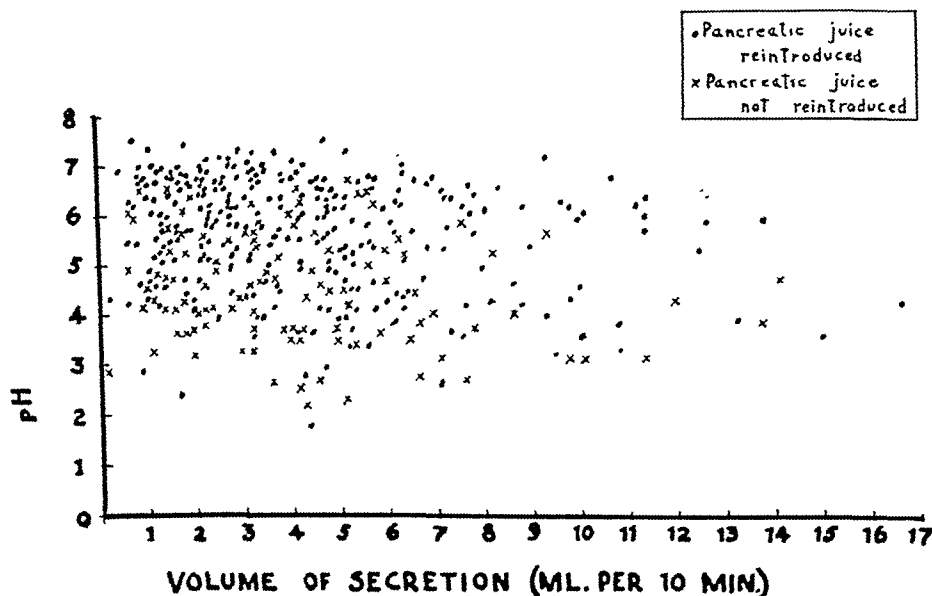


FIG. 2.

Scattergram showing the relationship between the pH of the duodenal content and the volume of pancreatic juice secreted during the ensuing 10-minute period.

minute period was plotted against the pH. Dots represent determinations made when the pancreatic juice was returned to the duodenum, crosses represent similar determinations made when the juice was withheld for the entire period of observation. The range of the pH was lower, but did not exceed the limits observed when the pancreatic juice was present in the duodenum. It is evident that the pancreatic juice exerts only a mod-

erate effect on the pH of the intestinal content.

The same data may be used to study the effect of acid in the intestine on the volume of pancreatic secretions. It is evident from a glance at this graph that no clear correlations between the volume of pancreatic secretion and the pH of the duodenal content existed in these experiments. This is rather strikingly illustrated by individual experi-

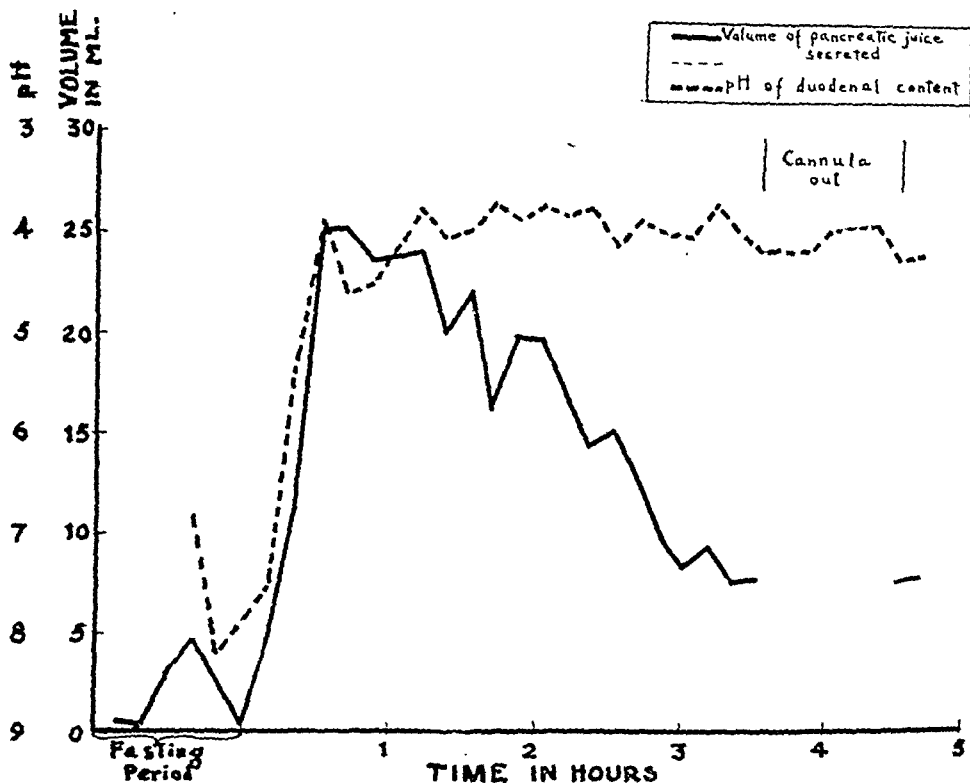


FIG. 3.

Curves of a typical experiment after the feeding of a large meal of meat, showing the relatively constant pH of the duodenal content in relation to the marked variation in the secretion of pancreatic juice at 10-minute intervals.

ments. Fig. 3 illustrates the results obtained when a large meal of meat was fed. It will be noted the pH of the duodenal content throughout the period of observation hovered about 4, whereas, the volume of pancreatic juice varied considerably. Fig. 4 represents data obtained after a small feeding of bread. It does not seem likely that the fluctuations in the volume of the pancreatic juice were related to the differences in pH observed.

Comment. Our observations are in conformity with those of Hoerner; namely, that some neutralization of the acid chyme is attained by the action of bile, succus entericus and other secretions of the small intestine and its appendages, but that generally more complete neutralization results when pancreatic juice is present in the intestine.

In addition, our findings would suggest that during digestion, the volume of pancreatic

juice secreted is determined by other factors than the acidity of the duodenal content alone. It would seem plausible that the secretin mechanism may "prime the pancreatic pump," since the duodenal acidity is at, or may exceed, the threshold value. However, other factors must play a determining role. Thus Crider and Thomas¹¹ found that, after section of the vagus nerves, the introduction of acid into the intestine was less effective in promoting pancreatic secretion than previously. Possibly two or more different stimuli must be active at any one time to evoke a copious pancreatic secretion, as does occur during the digestive period. However, it would seem that when the duodenal acidity exceeds a given threshold value, as does oc-

¹¹ Crider and Thomas, *Am. J. Physiol.*, 1944, 141, 730.

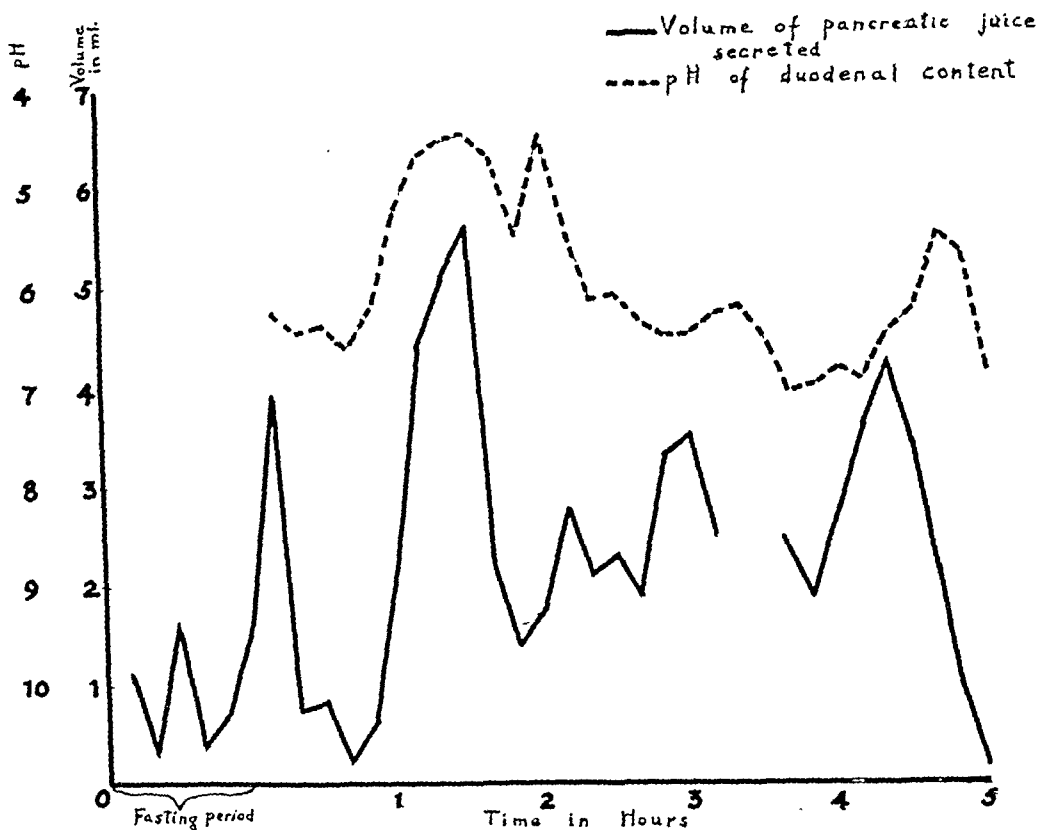


FIG. 4.

Curve showing a typical experiment after small feeding of bread, demonstrating the lack of relationship between the pH of the duodenal content and the secretion of pancreatic juice at any given time. Determinations were made at 10-minute intervals.

casionally occur, the secretin mechanism may call forth a larger volume of neutralizing pancreatic juice.

Conclusions. 1. Pancreatic juice provides buffering material which serves to neutralize gastric content more effectively, but some

degree of neutralization is attained from other secretions of the small intestine and its appendages.

2. No correlation is evident between the acidity of the duodenal content and the volume of pancreatic secretion.

In vitro Lysis of Leucocytes from Tuberculous Humans by Tuberculoprotein.*

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In a study on the delayed type of hypersensitivity, it has been found that lymphocytes from tuberculous mice and guinea pigs are specifically destroyed when exposed *in vitro* to tuberculoprotein.¹ The purpose of this report is to extend this type of observation to man.

Experimental. Using a method previously described,² suspensions of human white cells were obtained by layering whole, heparinized blood over albumin-saline mixtures,[†] and centrifuging. The resulting cell suspensions consisted of 50-80% lymphocytes, 20-40% neutrophil leucocytes, and small percentages of the other white blood cells. Experiments were performed on the leucocytes from 3 groups of subjects: (1) tuberculin-positive (PPD) patients with bacteriologically proven, active, severe tuberculosis; (2) subjects with positive tuberculin skin tests (PPD) but no demonstrable clinical tuberculosis; and (3) healthy persons with negative tuberculin skin tests (PPD, second strength).

Cytolysis was demonstrated by adding 0.2 cc of white cell suspension (7,000-15,000 cells per cu mm in fresh normal human serum) to 0.2 cc of "antigen" solution (fresh normal human serum containing PPD-s,[‡] 25 γ per cc). Directly after mixing, these preparations were incubated in a water-bath at 37°C.

* Work done under an U.S.P.H.S. Research Grant.

† Francis Weld Peabody Fellow in Medicine.

‡ Sterile 35% isotonic bovine albumin solution kindly supplied by Armour and Company, Stock Yards, Chicago, Ill.

1 Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.

2 Ferrebee, J. W., and Geiman, Q. M., *J. Inf. Dis.*, 1946, **78**, 173, as modified by Favour.¹

3 Seibert, F. B., and Glenn, J. T., *Am. Rev. Tub.*, 1941, **44**, 9.

Samples were taken at 5, 20, 60 (and 90) minutes for total white cell counts (5-10% error), and differential counts (1000 cells).

As cell system controls, parallel counts were performed on similar cell concentrations suspended in fresh normal human serum alone. Cell preparations suspended in fresh normal serum containing 200 γ per cc of a beta streptococcal protein, similar to that used to demonstrate cytolysis in tissue culture,⁴ served as "antigen" controls.

It was previously noted that phosphate or citrate ions blocked cytolysis in this type of experiment.¹ To further elucidate the role of complement in this reaction, fresh human serum heated at 56°C for one-half hour, was used in some experiments as the suspending and diluting fluid, in place of fresh normal human serum.

Experiments have been performed one or more times on at least 6 individuals in each of the 3 categories outlined.

Results. Sample protocols illustrate the results obtained in typical experiments.[§]

1. Twenty to 60% of the lymphocytes, and 20-90% of the neutrophil leucocytes from the peripheral blood of patients with active severe tuberculosis, are specifically destroyed within 90 minutes by contact *in vitro* with small amounts of tuberculoprotein (Table I).

2. If heat-inactivated serum is substituted for fresh normal serum as the suspending and diluting fluid in these preparations, no cytolysis occurs (Table I).

3. No cytolysis, beyond that induced by the trauma of the procedure (1-10% in 2 hours), occurs on exposure of these cells to

⁴ Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

[§] In the protocols, eosinophils, basophils, and monocytes have not been recorded as they total less than 2%.

TABLE I.
In vitro Effect of PPD on Blood Leucocytes from a Patient with Miliary Tuberculosis (Tuberculin Positive).

		Total WBC	Lymphocytes		Granulocytes	
			%	Absolute No.	%	Absolute No.
PPD	5'	2430	77.1	1880	21.8	530
Normal serum	30'	1260	88.1	1080	11.0	136
	60'	810	87.5	700	11.4	92
PPD	5'	3190	66.2	2110	33.6	1070
Heated serum	30'	3270	65.2	2130	34.8	1140
	60'	3280	70.0	2290	30.0	990
Normal serum	5'	2580	78.4	2020	21.3	550
control	30'	2380	79.0	1880	20.1	480
	60'	2570	79.7	2050	19.6	500

Note: Progressive cytolysis of lymphocytes and granulocytes in the presence of PPD and fresh normal human serum. No cytolysis with heat-inactivated serum.

TABLE II.
In vitro Effect of PPD on Blood Leucocytes from a Patient with Tuberculous Pneumonia (Tuberculin Positive).

		Total WBC	Lymphocytes		Granulocytes	
			%	Absolute No.	%	Absolute No.
PPD	5'	8890	53.2	4730	46.6	4140
Normal serum	20'	8130	52.0	4220	47.7	3880
	60'	7220	56.6	4080	43.3	3120
	90'	6160	52.4	3220	46.9	2890
Strep. protein	5'	8130	57.4	4670	42.2	3430
Normal serum	20'	8440	56.0	4730	44.0	3710
	60'	8060	60.0	4840	40.0	3220
	90'	8120	56.4	4580	43.2	3500
Normal serum control	5'	7790	58.0	4520	41.6	3240
	20'	7450	59.0	4400	41.0	3050
	60'	7030	60.0	4220	40.0	2810
	90'	7320	58.2	4260	41.6	3050

Note: Progressive cytolysis of lymphocytes and granulocytes in the presence of PPD and fresh normal human serum. No cytolysis with strep. protein (200 γ per cc).

(a) fresh normal human serum alone, or
(b) streptococcal protein in fresh normal serum (Table II).

4. Leucocytes from healthy tuberculin-positive or tuberculin-negative subjects are not affected by contact *in vitro* with PPD or streptococcal protein, under the conditions of these experiments.

Conclusions. The observation that lymphocytes from animals infected with certain bacteria are specifically destroyed by contact *in vitro* with protein extracts of these bacteria¹ has been extended to humans infected with *M. tuberculosis*. In the human, as in the

guinea pig, the neutrophil leucocytes are also specifically lysed. It is of interest that although the lymphocytes from tuberculous subjects of all 3 species (mouse, guinea pig, human) so far studied, are specifically sensitive to tuberculo-protein, the neutrophil leucocytes are affected only in those species which show a positive delayed-type tuberculin skin test (*i.e.*, guinea pigs and humans). In the tuberculous mouse, which fails to develop delayed-type skin hypersensitivity reactions to tuberculo-protein,⁵ lympholysis occurs but

⁵ Gerstl and Thomas, *Yale J. Biol. and Med.*, 1940-41, 13, 679, ref. cit.

other cell types remain unaffected.¹ Apparently, the presence of complement is necessary for the occurrence of this form of prompt cytolysis. Experiments to further elucidate

the role that this type of cytolysis plays in the pathogenesis of delayed-type hypersensitivity are in progress.

16354 P

An Electrophoretic Study of the Serum Proteins in Scleroderma.

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Scleroderma (literally: "hardening of the skin") occurs in two forms: diffuse and localized. The diffuse form is a progressive systemic disease of the connective tissue throughout the body.^{1,2} The localized form on the other hand appears clinically to be limited to the skin and is a much milder disease. In spite of numerous investigations, the pathogenesis of scleroderma remains obscure. Observations on the serum proteins have been limited to an occasional routine determination showing a decrease in the albumin/globulin ratio with little change in the value for total proteins.³ It seemed possible that a more detailed analysis by the Tiselius method of electrophoresis might yield additional information of value.

Methods and Materials. Serum was obtained in the fasting state from 12 normal subjects, 5 patients with diffuse scleroderma, and one patient with localized scleroderma. Four ml of serum were dialyzed against 2 liters of barbiturate buffer (ionic strength 0.1, pH 8.6) for 48 hours at 4°C, then diluted 1:3 with buffer. Electrophoresis was carried out in the Tiselius apparatus using a double section cell and an optical system of the type described by Philpot.⁴ It proceeded for 80 minutes at a potential gradient of 8 volts per

cm and a temperature of 1°C. Patterns were photographed directly, enlarged 5X, and the areas measured with a planimeter. Components were delineated by vertical lines drawn from the minima of the curves to the baseline. Ascending and descending pattern areas were averaged for each component except β -globulin; here only the ascending pattern area was used. Total and nonprotein nitrogen were determined by the micro-Kjeldahl method.

Results. In diffuse scleroderma, as shown in Table I, the albumin fraction of the serum proteins decreases and the γ -globulin fraction increases, but no significant change occurs in the total protein value. The alterations in the albumin and γ -globulin fractions are statistically significant. No new components in the electrophoretic pattern and no gross alterations in mobilities are evident. While little weight can be given to data from one case of localized scleroderma, it is interesting to note that the changes are qualitatively and quantitatively similar to those occurring in the diffuse scleroderma group. This is somewhat surprising in view of the apparent absence of systemic involvement in the localized form of the disease.

Discussion. The changes described are in no way diagnostic since similar changes occur in many chronic infections, in disseminated lupus erythematoses, in liver disease, and in other conditions.⁵ They may, however, give

¹ Matsui, S., *Press. med.*, 1924, **2**, 142.

² Lindsay, J. R., Templeton, F. E., and Rothman, S., *J. A. M. A.*, 1943, **123**, 745.

³ Banks, B. M., *New Eng. J. Med.*, 1941, **225**, 433.

⁴ Philpot, J. St. L., *Nature*, 1938, **141**, 283.

⁵ Stern, K. G., and Reiner, M., *Yale J. Biol. and Med.*, 1946, **19**, 67.

TABLE I.
Electrophoretic Analysis of Serum Proteins in Scleroderma.

Electrophoretic Analysis of Serum Proteins in Scleroderma.											
Patient No.	% of total protein					Concentration, g %					Total protein
	Alb.	Globulins				Alb.	Globulins				
		α_1	α_2	β	γ		α_1	α_2	β	γ	
Diffuse Scleroderma.											
1	44.5	4.6	10.3	17.2	23.4	3.30	0.34	0.76	1.27	1.73	7.41
2	37.9	4.6	9.1	14.3	14.1	3.66	0.29	0.58	0.90	0.89	6.32
3	47.1	6.0	9.1	12.6	25.3	3.52	0.45	0.68	0.94	1.89	7.47
4	44.5	4.6	10.3	17.2	23.4	3.19	0.33	0.74	1.23	1.68	7.17
5	44.4	7.9	7.9	17.6	22.0	2.36	0.42	0.42	0.94	1.17	5.31
Mean	47.7	5.5	9.3	15.8	21.6	3.21	0.37	0.64	1.06	1.47	6.74
σ	5.82	1.45	1.00	2.22	4.38	0.508	0.067	0.140	0.178	0.422	0.921
Localized Scleroderma.											
6	46.9	5.4	8.0	12.0	27.6	3.64	0.42	0.62	0.93	2.14	7.77
Normal (12 subjects).											
Mean	58.4	4.7	9.2	15.2	12.8	4.00	0.32	0.63	1.05	0.89	6.90
σ	2.81	0.67	1.00	1.95	2.45	0.276	0.039	0.091	0.162	0.207	0.483

σ = Standard deviation.

TABLE II.
Clinical Data on the Patients.

Patient No.	Age	Sex	Duration of disease, yrs	Tissues involved in addition to skin	Course
1	25	F	3		Stationary
2	36	F	2	Joints	"
3	54	M	7	Esophagus, lungs, joints, pleura, pericardium, connective tissue (generalized)	Expired (autopsy)
4	58	M	2½	Joints	Expired
5	62	M	2	Esophagus, lungs, joints, renal and myocardial vessels*	" (autopsy)
6	13	F	6		Progressive

* Patient 5 also showed hypertension. Scleroderma was the only disease noted in any of the other patients.

some clue as to the pathogenesis of scleroderma. The rise in the γ -globulin fraction, which contains most of the known antibodies, suggests an immunological response, such as that which occurs during immunization,⁶ or in the course of infectious diseases. Decrease

in the albumin fraction, which occurs early in scleroderma, is also a characteristic of the electrophoretic pattern in malnutrition, liver disease and kidney disease.⁵ Liver involvement is seldom found at autopsy in scleroderma, but kidney changes do occur, and a poor state of nutrition commonly accompanies the disease.

⁶ Van der Scheer, J., Bohnel, E., Clark, F. H., and Wyckoff, R. W. G., *J. Immunol.*, 1942, 44, 165.

Effect of Acute Liver Damage on Ac-Globulin Activity of Plasma.*

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Normal plasma has been shown to contain a factor, Ac-globulin, which is a highly active accelerator of blood clotting.¹⁻³ It acts by catalyzing the interaction of prothrombin, thromboplastin and calcium with the resultant formation of thrombin. The hemorrhagic disease, parahemophilia, recently reported to occur in the human being,⁴ is the result of a deficiency of this factor. Ac-globulin is differentiated from prothrombin in that it is precipitated from aqueous solutions at a higher pH, is more readily destroyed by heating, and is less soluble in concentrated ammonium sulfate solutions.^{3,5} It is measured quantitatively⁵ by an adaptation of the 2-stage method of prothrombin determination and may be detected in normal dog plasma in dilutions up to 350,000.

In acute liver damage, such as that produced by the administration of chloroform, there is a marked decrease in plasma prothrombin and fibrinogen.^{6,7} In this paper we report that acute liver damage produced by chloroform also lowers plasma Ac-globulin.

Experimental. Quantitative estimation of Ac-globulin was achieved by an adaptation of the 2-stage prothrombin assay procedure. Details of the method are reported elsewhere.⁵ In essence the method consists of comparing

the rates of activation of prothrombin with thromboplastin and calcium ions in the presence of known and unknown amounts of Ac-globulin. The prothrombin, thromboplastin and calcium are kept constant. When prothrombin is activated in this manner under standard conditions the rate of thrombin formation is directly proportional to Ac-globulin concentration. Thrombin is measured by adding fibrinogen at intervals and noting the clotting time.

Three adult male dogs weighing 13 to 19 kg were observed for a period of 2 weeks and judged to be healthy on the basis of normal rectal temperature, maintenance of weight and food intake, alertness and activity. They were fasted for 36-72 hours and then given moderately deep chloroform inhalation anesthesia varying in length of time from 1½ to 3 hours. Plasma Ac-globulin, coagulation time (Lee-White), hematocrit and icterus index were determined at intervals before and after chloroform administration. Prothrombin was determined by the 2-stage method of Warner, Brinkhous and Smith,^{6,8} as modified by Ware and Seegers.⁵ The modification consists only of supplying Ac-globulin when necessary. Heparin was used as the anticoagulant for blood specimens taken from one dog and 3.5% sodium citrate for those taken from the other 2 dogs.

Results. For 4 to 6 days following the administration of chloroform, the dogs showed lethargy, anorexia, and a low-grade temperature increase. The establishment of liver damage was shown by the development of an icteric color to the plasma first becoming noticeable 24-48 hours after the anaesthesia, and by the development of hypoprothrombinemia. At no time, however, did the animals show clinical jaundice or clay-colored stools.

* Aided by a grant from the U. S. Public Health Service.

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2 Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

3 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

4 Owren, P. A., *Lancet*, 1947, **252**, 446.

5 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

6 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

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8 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

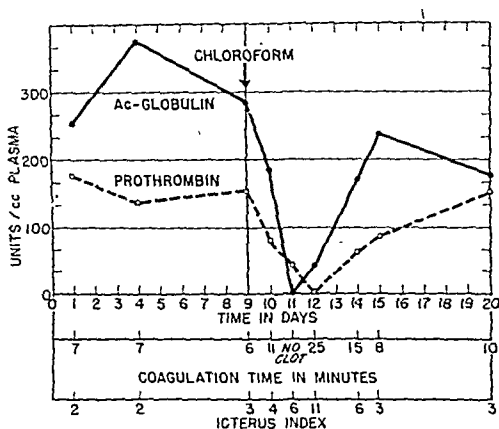


FIG. 1.

The effect of acute liver damage (chloroform intoxication) on the plasma Ac-globulin concentration of the dog.

The onset of the plasma jaundice and the peak of the icterus index rise were not associated with a drop in the hematocrit. All of the dogs survived.

The duration of chloroform administration, from $1\frac{1}{2}$ to 3 hours, appeared to have no marked influence on the resulting changes in Ac-globulin and prothrombin concentrations. The values shown in Fig. 1 for the dog anesthetized for a period of 3 hours are representative for the group. Within 24 to 48 hours after anaesthesia, the plasma Ac-globulin decreased precipitously in all dogs from initial values of 100 to 370 units per cc to

low values of 1 to 3 units per cc. Prothrombin also decreased to low levels but did so somewhat later, within 48 to 72 hours after anaesthesia. Ac-globulin returned to normal values in approximately 6 days, while prothrombin returned more slowly, reaching normal values in about 10 days. Significantly increased coagulation time, hematoma formation and excessive bleeding from venipuncture wounds were encountered 2 days after anaesthesia; the dog receiving 3 hours of anaesthesia, in addition, showed continuation of the bleeding tendency through the third day. Fibrinogen was absent in this dog 2 days after anaesthesia as shown by the failure of blood samples to clot following the addition of a very active preparation of thrombin.

Discussion. These experiments indicate that the hemorrhagic tendency occurring in cases of acute liver damage is attributable not only to decreased prothrombin and fibrinogen concentration^{6,7} but also to decreased plasma Ac-globulin. The data suggest further that plasma Ac-globulin is formed in the liver.

Summary. Acute hepatic damage in the dog, caused by chloroform intoxication, is followed by a pronounced and rapid decrease in plasma Ac-globulin concentration. This decrease is accompanied by a parallel decrease in plasma prothrombin concentration. Ac-globulin returns to normal values more quickly than does prothrombin.

Prevention by Sodium Salicylate of Arteritis in the Experimental Allergic State.*

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(Introduced by Ralph A. Kinsella.)

(With the technical assistance of Helen Lee.)

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It has been established that arteritis can be produced in the experimental animal following the injection of foreign protein.¹⁻⁶ We have attempted to produce experimental arteritis in one group of rabbits and to prevent its expected production in a similar group, with the purpose of clarifying the mechanism of its pathogenesis. Several extensive reviews of the problem have appeared, among which is that of Logue and Mullins.⁷ The pathology has been described, and circulating antibody has been studied.^{1,4,6,8,9,10,11} The process which precedes antigen-antibody reaction in the fixed tissues has in part been indirectly demonstrated¹²⁻¹⁵ by the recovery of humoral antibody against homologous kidney from

animals injected with hemolytic streptococci; this suggests initial union of the streptococcus antigen with a kidney tissue hapten fraction. A histologic correlation with the immunological process in experimental arteritis⁶ has shown that one of the earliest manifestations of the lesion is collection of lymphocytes and monocytes around the artery at which time circulating antibody is absent. These observers⁶ present these findings as evidence of fixation of antigen in the arterial wall, and of antibody migration within the lymphocytes to the site of fixed antigen.

Sodium salicylate suppresses circulating antibody in the experimental animal^{1,16,17} and in clinical serum sickness.⁹ Given intravenously¹ it did not prevent the occurrence of experimental arterial lesions. Suppression of circulating antibody was reconciled with hastening of recovery by postulating that the same mechanism which rendered an infectious agent less antigenic might also render it less pathogenic.¹⁶ Sodium salicylate *in vitro* exerts some inhibitory effect on immune reactions.^{18,19} Some investigators have found

* This work was aided by funds from the James B. Miller Institute of Experimental Medicine of St. Louis University.

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² Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, **72**, 65.

³ Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 239.

⁴ Fox, R. A., and Jones, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 294.

⁵ Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. Clin. Med.*, 1947, **32**, 379.

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⁸ Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, **27**, 341.

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¹³ Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 158.

¹⁴ Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 163.

¹⁵ Jones, L. R., personal communication.

¹⁶ Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

¹⁷ Homburger, F., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 101.

¹⁸ Murrack, J., and Smith, F. C., *Brit. J. Exp. Path.*, 1931, **12**, 30.

¹⁹ Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

²⁰ Guerra, F., *Science*, 1946, **103**, 686.

in vitro and *in vivo* inhibition of hyaluronidase activity by salicylates^{20,21} and believe this is due to inhibition of the enzyme reaction involved in liberation of N-acetyl-glucosamine. The toxic effects of salicylates have been studied^{22,23,24} and are proportional to size of dosage.

Anti-histaminic drugs protect against anaphylaxis²⁵ but there is no correlation between anaphylactic reactions and the occurrence of vascular lesions.⁴ However, the anti-histaminic drugs greatly minimize the vascular lesions in experimental arteritis⁵ and do have an anti-hyaluronidase action.²⁶ Hyaluronidase greatly intensifies the size of epidermal allergic reactions.²⁶

Para-aminobenzoic acid is apparently concerned with metabolic stimulation of host cells.²⁷

Method. Twenty albino rabbits were given intravenous injections of sterile horse serum on 3 occasions. The first dose was 10 cc per kilo; the second dose, given 16 days later, was one cc per animal; the third dose, given 6 days later, was 10 cc per kilo. Seven days after the third injection all surviving rabbits were sacrificed by air emboli and were immediately autopsied. The organs were fixed in formalin, sectioned, stained, and the heart and kidneys carefully studied for the presence of arteritis.

The animals were divided into 4 groups.

Group A, 3 rabbits, was given para-aminobenzoic acid starting 31 days prior to the first injection of horse serum and continuing throughout the course of the experiment. The PABA was dissolved in their drinking water,

each cc containing 0.0026 g of PABA.

Group B, 12 animals, was given sodium salicylate daily, starting 8 days prior to the first injection of horse serum and continuing throughout the course of the experiment. A 5% aqueous solution was employed and the injections were given subcutaneously once each day. The daily dose was 0.2 g per kilo of body weight.

Group C, 2 animals, was treated exactly the same as Group B with the exception that each injection of sodium salicylate was given intravenously prior to the first injection of horse serum. After that all sodium salicylate injections were given subcutaneously.

Group D, 3 animals, received nothing other than horse serum.

The animals in all groups were bled on one or several occasions and the serum was tested for the presence of precipitin against horse serum.

Results. The results are shown in Table I. The 3 animals in Group B that failed to survive died during the night in the last week of the experiment. In the presence of post-mortem changes no conclusions can be drawn from the appearance of their tissues. However all 3 showed the same findings, consisting of gangrene of the caecum, peritonitis, and lymphocytic and monocytic infiltration around the arteries of the kidneys. Group C animals both showed, in addition to periarterial round cell infiltration, diffuse and localized collections of lymphocytes throughout the parenchyma of the kidney, thickening of some glomerular membranes, and infiltrations of mononuclear cells around the afferent arteriole.

Discussion. Whereas the horse serum control animals and 2 of the PABA treated animals developed arterial lesions and circulating antibody at the end of the experiment, the findings were different in the sodium salicylate treated animals. Moreover, the route of administration of the sodium salicylate apparently influenced the effect. When it was given subcutaneously the surviving animals showed no evidence of arteritis but did produce circulating antibody in moderate or attenuated quantity, late in the course of the experiment.

²¹ Dorfman, A., Reimers, E. J., and Ott, M. L., 1947, **64**, 357.

²² Stevenson, C. S., *Am. J. Med. Science*, 1937, **193**, 772.

²³ Guest, G. M., Rapoport, S., and Roscoe, C., *J. Clin. Invest.*, 1945, **24**, 770.

²⁴ Jager, B. V., and Alway, R., *Am. J. Med. Science*, 1946, **211**, 273.

²⁵ Mayer, R. L., Hutterer, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

²⁶ Mayer, R. L., and Kull, F. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 392.

²⁷ Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

When it was given intravenously first and subcutaneously later the animals did develop arteritis and glomerular changes, but produced circulating antibody in scarcely detectable quantity, late in the course of the experiment.

The following theory is offered as a possible explanation of the reactions here observed. When sodium salicylate is administered subcutaneously sufficiently in advance of the first injection of antigen, it blocks the fixation of antigen in the tissue cells. This precludes any need of antibody in the tissues and there is, therefore, no lymphocytic or monocytic migration to the tissues. In this absence of tissue antigen-antibody union the chain of events leading to production of lesions is never started. The intravenously injected antigen circulates for a prolonged period, and in its passage through the lymph nodes and other reticulo-endothelial structures gives rise to production of antibody. Union of this circulating antibody with its antigen is prevented by reason of the inability of antigen to become fixed to tissue cells. The presence of this circulating antibody thus becomes an indifferent matter to the well being of the host, at least insofar as this type of allergic reaction is concerned. The observations of others concerning the potentiating effect of

hyaluronidase in a certain type of allergic reaction²⁰ and the anti-hyaluronidase activity of sodium salicylate,^{20,21} suggest that sodium salicylate blocks antigen fixation in the tissues by inhibiting hyaluronidase activity.

No conclusions can be drawn concerning the effect produced when sodium salicylate is given intravenously prior to the first injection of the antigen and subcutaneously thereafter, since only 2 animals were treated in this manner. However, the identical findings in both suggest a lowering of the protective action against antigen-tissue fixation, and a heightening of the depressant effect on the reticulo-endothelial system. This problem is at present under further investigation.

Conclusions. 1. The subcutaneous administration of large doses of sodium salicylate to rabbits well in advance of the initial contact with horse serum antigen prevents the development of arterial lesions.

2. The arterial lesions fail to develop even though circulating antibody is present in moderate quantity.

3. It is believed that the lesions fail to develop because there is no antigen-antibody reaction, and that this reaction can not take place because salicylate has prevented antigen from uniting with tissue cells.

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Specificity of the Response of Various Assay Organisms to Nicotinic Acid.¹²

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It has recently been shown with *Neurospora* mutants that tryptophan can be converted to nicotinic acid and that kynurenine and hydroxyanthranilic acid are intermediates in

the conversion.^{1,2} This conversion, which also appears to be carried out by the intestinal flora of animals,^{3,4} and possibly by animal tis-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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¹ Beadle, G. W., Mitchell, H. K., and Nye, J. F., *Proc. Nat. Acad. Sci.*, 1947, **33**, 155.

² Mitchell, H. K., and Nye, J. F., *Proc. Nat. Acad. Sci.*, 1948, **34**, 1.

³ Ellinger, P., and Abdel Kader, M. M., *Nature*, 1947, **160**, 675.

sues,[†] is of considerable interest in human nutrition.[‡] Since microorganisms are widely used for the determination of nicotinic acid, and since these intermediates occur naturally under some conditions, it was of interest to determine whether the commonly used assay organisms could utilize *l*-kynurenine or hydroxyanthranilic acid for growth in place of nicotinic acid. For the organisms tested, these compounds were uniformly inactive. The data essential for establishing this fact are presented below.

Experimental. Cultures and Methods. The cultures used were *Lactobacillus arabinosus* 17-5, *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R, *Proteus vulgaris* and *Torula cremoris* 2512. The niacin assay medium employed with the first three organisms was the complete amino acid assay medium described by Henderson and Snell,[§] with niacin omitted. For *Proteus vulgaris*, the medium of Saunders *et al.*[‡] was used. With *Torula cremoris*, the medium and assay conditions described by Williams[§] were employed. In all cases, solutions of *l*-kynurenine and hydroxyanthranilic acid[§] were sterilized by filtration, and added aseptically to the basal media after these had been sterilized by autoclaving. The final volume in all assay tubes was 2 cc. After a three-day incubation period, which permitted heavy growth of all organisms except *Proteus vulgaris* in media containing excess niacin, cultures were diluted

[‡] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1946, **163**, 343.

[†] Cf. the synthesis of niacin which occurs during incubation of hen (Quarles, E., and Snell, E. E., *J. Nutrition*, 1941, **22**, 483) and turkey (Furman, C., Snell, E. E., and Cravens, W. W., *Poultry Science*, 1947, **26**, 307) eggs, where no microorganisms are present.

[§] Anonymous, *Nutrition Rev.*, 1947, **5**, 247.

[§] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

[‡] Saunders, F., Dorfman, A., and Koser, S. A., *J. Biol. Chem.*, 1941, **138**, 69.

[§] Williams, W. L., *J. Biol. Chem.*, 1946, **166**, 397.

[§] We are indebted to Dr. H. K. Mitchell for samples of *l*-kynurenine and hydroxyanthranilic acid. Synthesis of the latter compound has been described recently (Nye, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, in press).

TABLE I.
Growth Response of Various Organisms to *l*-Kynurenine and Hydroxyanthranilic Acid in Presence and Absence of Nicotinic Acid.*

Substance added γ per 2 cc. Organism	Nicotinic acid		<i>l</i> -Kynurenine		Hydroxyanthranilic acid				Hydroxyanthranilic acid plus kynurenine		Niacin (0.5 γ) plus hydroxyanthranilic acid (0.5 γ) plus kynurenine (0.5 γ)	
	0	.05	0.1	10	0.1	10	0.1	10	0.5 γ each	0.5 γ each		
<i>L. arabinosus</i>	91	70	91	97	95	94	95	94	95	95	74	74
<i>Leuc. mesenteroides</i>	95	74	95	96	96	96	96	96	98	98	76	76
<i>S. faecalis</i>	92	79	92	92	92	92	92	92	92	92	80	80
<i>P. vulgaris</i>	99	92	99	99	99	99	99	99	99	99	92	92
<i>T. cremoris</i>	99	77	99	99	97	99	97	99	96	96	76	76

* Several levels of each substance in addition to those shown were tested. In no case was evidence of any growth-promoting action apparent.

with water to 10 cc and turbidity values determined with the Evelyn photoelectric colorimeter. Results are given in Table I. In no case did kynurenine or hydroxyanthranilic acid, either singly or combined, in the presence or absence of sub-optimal quantities of niacin, enhance growth. Ornithine, glutamine and arginine, which increase nicotinamide production by some strains of *E. coli*, have also been shown to be ineffective in substituting for the niacin requirement of *L. arabinosus*,³ the organism most commonly used for assay of this vitamin.

Summary. Kynurenine and hydroxyanthranilic acid, which appear to be intermediates in the conversion of tryptophan to nicotinic acid by *Neurospora*, neither replace nicotinic acid nor enhance the growth response to sub-optimal amounts of nicotinic acid for *L. arabinosus*, *Leuc. mesenteroides*, *S. faecalis*, *Proteus vulgaris*, or *Torula cremoris*. These substances do not, therefore, interfere in microbiological assays for niacin which employ these test organisms.

Appreciation is expressed to Professor Ch. Weizmann whose kindness made this study possible.

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Paper Partition Chromatographic Analysis and Microbial Growth Factors: The Vitamin B₆ Group.

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Microbial growth factors often exist in nature in more than one chemically defined form. In the past, differential assays with several microorganisms have been used to demonstrate this multiplicity.

The present report deals with an alternate procedure for recognizing the presence of different forms of a growth factor. The method involves the combined use of paper partition chromatography and a microbial indicator and represents, as far as the writers are aware, a new methodological principle for the recognition and resolution of different chemical forms of a growth factor.

As will be seen, the method to be described is the counterpart of the technique used in the differentiation of the several forms of penicillin by Goodall and Levi¹ and independently by Winsten and Spark² except that in dealing with growth factors, zones of microbial growth serve to delineate the different members of a complex instead of zones of inhibition, as in

the case of the antibiotics. The use of zones of microbial growth to assay vitamins was suggested by the work of Mager and Aschner.³

In the present method, the underlying principle involves the separation on a paper strip chromatogram of the different chemical forms of a growth factor. After the separation has been achieved, the positions of the different forms on the paper strip are revealed by the use of a microbial indicator, that is, a microorganism capable of utilizing at least one and usually several forms of the factor for growth. The positions of the various chemical entities on the chromatogram serve to characterize and identify the particular substances present. This is a consequence of the fact that the distance each substance moves is related to its specific partition coefficient for the solvents employed in developing the chromatogram.

In what follows, the application of this method to the resolution and identification of the members of the B₆ group will be described. This particular complex of growth factors was

¹ Goodall, R. R., and Levi, A. A., *Nature*, 1946, **158**, 675.

² Winsten, W. A., and Spark, A. H., *Science*, 1947, **106**, 192.

³ Mager, J., and Aschner, M., *J. Bact.*, 1947, **53**, 293.

studied first since its component parts are well known, principally due to the work of Snell.⁴ The present report is intended to demonstrate the potentialities of the technique employed and to describe some results obtained by its use.

Method. In applying the method to the vitamin B₆ group, a 0.007 ml drop of a solution (pH adjusted to 5.0) containing 1-5 μ g per ml of each of the various members of the group is applied near the head of a paper strip chromatogram (Whatman No. 4 paper strips 1" by 16" are used). The chromatogram is then developed for about 6 hours with wet butanol in a humid atmosphere in the manner first devised for amino acids by Consden, Gordon and Martin.⁵ After drying for 15 minutes at 65°C, the paper strip chromatogram with the various members of the vitamin B₆ complex now occupying definite positions along the strip, is laid on an agar plate, seeded with *Saccharomyces carlsbergensis*, strain 4228, an organism which exhibits a growth response to all three known forms of the vitamin. The nutrient agar⁶ contains all factors necessary for the growth of the organism with the exception of the vitamin B₆ group. In making the agar plate a bottom layer of the nutrient agar is made by pouring 300 ml of the agar medium on a plate 11" by 18". This is allowed to harden. A 200 ml portion of the nutrient agar cooled to 48-50°C is seeded with 10 ml of sterile physiological saline to which has been added a loopful of a 24-hour culture of the yeast. The seeded agar is then poured on the hardened underlayer and allowed to cool. (Using such a plate, as many as 8 strip chromatograms resulting from eight separate analyses may be laid parallel to each other on the nutrient agar at one time). The paper strip chromatogram is allowed to soak for 5 minutes on the surface of the moist agar in order to transfer the various B₆ members from the strip to the

agar plate. The strip is then removed leaving its imprint, however, on the agar surface. The agar plate is then incubated overnight at 27-30°C. After incubation well defined elliptical zones of growth of the organism are seen at various intervals along the locus of the chromatogram marking the loci of the various members of the vitamin B₆ group. The area of each zone of growth is a measure of the amount of the particular substance causing the growth. While it has proved possible to obtain a dose response curve for each substance by plotting area of growth against weight of substance per ml, the greatest utility of the method lies in its ability to resolve and identify the various members in complex mixtures of the different forms of vitamin B₆. Visual inspection of the size of the zones of growth gives a graphic picture of the relative amounts of the various forms present in a mixture.

The different members of a complex are identified by their relative positions along the length of a chromatogram. Each substance has its characteristic R_F value which is defined according to Consden *et al.*⁵ as the ratio of the distance a substance has moved down the chromatogram to the distance the developing solvent has traveled. This is conveniently measured directly on the agar plate if one imprints on the agar the spot where the original drop was applied as well as the boundary on the strip to which the solvent front has moved. This is readily done when the paper strip is still in place on the agar surface. The R_F value is then equal to the distance of the center of a zone of growth from the point of application of the sample being analyzed, divided by the distance the solvent has moved measured from the point of application of the drop.

Results. The above technique was first applied to the resolution of the members of the vitamin B₆ group in a synthetic mixture.

On chromatographing a 0.007 ml sample of a mixture containing 5 micrograms each per ml of pyridoxal, pyridoxine and pyridoxamine with wet butanol, the characterizing R_F values for the component factors were found to be: pyridoxamine 0.18, pyridoxal

⁴ Snell, E. E., *J. Am. Chem. Soc.*, 1944, **66**, 2082.

⁵ Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

⁶ Atkin, L., Schultz, A. S., Williams, S. L., and Frey, L. M., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.



FIG. 1.

Results of paper partition chromatography applied to synthetic members of the B_6 group. The photograph represents a picture of a typical agar plate and shows the zones of growth of *S. carlsbergensis*, Strain No. 4228, along the loci of the paper strip chromatograms. The symbol (a) refers to the locus of point of application of the sample. The symbol (b) refers to the boundary to which the solvent had moved. The strips represent the result of chromatographing a mixture of pyridoxal, pyridoxamine, and pyridoxine (strip 1), pyridoxamine alone (strip 2), and pyridoxal alone (strip 3), and pyridoxine alone (strip 4).

0.68, pyridoxine 0.75. That is, pyridoxamine moves least rapidly on the chromatogram. Fig. 1 shows the results of such an experiment.

The R_F values were found to vary 10 to 20% from day to day. However, on any given day replicate chromatograms gave self-consistent R_F values. The relative positions on the chromatograms of the different forms of B_6 remained the same.

In order to simulate what might occur on

chromatographing an extract of the vitamin B_6 group obtained by the usual acid hydrolysis of natural products, a study was then made of the influence of a mixture of amino acids on the R_F values of the B_6 group members since various amino acids might be expected to be present in such extracts. Accordingly, the various forms of B_6 dissolved in a solution containing 5 per cent casein hydrolysate adjusted to pH 5, were chromatographed as above.

The presence of the amino acids caused a diminution in the R_F value of pyridoxamine from 0.18 to 0.06; that for pyridoxine decreased from 0.75 to 0.64.

Since the presence of amino acids in solutions being analyzed for the B_6 group alters the R_F values, these are not sufficient to identify the B_6 components unequivocally in an unknown mixture. It has been found expedient to identify members of the B_6 group in an extract of natural origin by obtaining a second chromatogram of the sample to which a known form of B_6 has been added to serve as a marker. The relative position occupied by the various B_6 members to that of the known form of B_6 added as a marker, serve to identify the former.

The results obtained on chromatographing a solution of pyridoxal and casein hydrolysate amino acids indicated that pyridoxal had reacted with certain constituents of the hydrolysate. Two zones of growth were observed. One was identified as pyridoxamine. On standing for several days at icebox temperature under aseptic conditions the pyridoxamine zone increased in size indicating that almost half the original pyridoxal was converted to pyridoxamine. Snell and Rannefeld⁷ have shown that on heating pyridoxal in solutions of amino acids, it is converted to pyridoxamine. However, the ease with which this reaction occurs, even at low temperatures, has not been fully appreciated. This finding throws some doubt on the validity of the usual type of microbiological assay for pyridoxal where the extract also contains amino acids, since these might convert an unknown frac-

⁷ Snell, E. E., and Rannefeld, A. H., *J. Biol. Chem.*, 1945, **157**, 475.

tion of pyridoxal to pyridoxamine.

The second zone observed on chromatographing pyridoxal in casein hydrolysate solution, namely that due to pyridoxal itself, was found to be more diffuse and cover a larger area along the locus of the chromatogram. This lengthening of the zone was interpreted as being due to a reversible reaction of pyridoxal and certain amino acids, a fact suspected by Snell but not heretofore easily demonstrated.

In using the analytical technique under discussion it has been possible to demonstrate a heretofore unrecognized reaction of a reversible type between *L*-cysteine and pyridoxal. This reaction which is non-enzymatic, occurs merely on incubating together a concentrated solution of cysteine and pyridoxal. The reaction is to be distinguished from that occurring between pyridoxal and other amino acids such as glutamic acid to yield pyridoxamine. In addition to a small but variable amount of pyridoxamine which does form during the reaction, there is an additional substance formed which moves more slowly on the chromatogram than does pyridoxamine. A typical experiment in which pyridoxal and cysteine were allowed to react was conducted as follows: 179 mg of pyridoxal hydrochloride and 157 mg of cysteine hydrochloride were dissolved in one ml of water. After standing overnight at 37°C the solution was diluted to 5 ml and 0.16 ml of pyridine was added to neutralize the acidity. The solution was then allowed to stand 2 days at room temperature. After appropriate dilution to yield solutions containing the nominal amount of 360 μ g per ml and 36 μ g per ml of pyridoxal hydrochloride based on the original amount of pyridoxal hydrochloride used, and adjustment to pH 5.0, 0.007 ml samples were taken for chromatographic analysis. The results of this analysis appear in Fig. 2. An examination of the replicate chromatograms 3 and 4 obtained for the more concentrated sample, reveals two zones of growth connected by a band of growth. The lower zone is due to the faster moving unchanged pyridoxal. The upper zone is due to a new substance which moves on the chromatogram at a rate less than that of



Fig. 2.

Results of paper chromatographic analysis of a reaction mixture of *L*-cysteine and pyridoxal. Duplicate strips 1 and 2 represent the analyses of a 0.007 ml sample of a solution containing the equivalent of 36 micrograms of pyridoxal hydrochloride per ml. Duplicate strips 3 and 4 are those obtained for a solution containing the equivalent of 360 μ g per ml of pyridoxal hydrochloride.

pyridoxamine itself as established in a separate experiment. The new zone of growth probably represents an adduct formed from cysteine and pyridoxal and is either a new form of vitamin B₆ or a substance which readily regenerates the original pyridoxal for use by the microorganism. The reaction to yield the substance in question is evidently reversible. Such reversibility would explain the band of growth connecting the two zones since, as the solvent used to develop the chromatogram moves the pyridoxal down the column, its removal from the original point of application of the sample will reverse

the equilibrium reaction of cysteine and pyridoxal regenerating the latter; this would then make its appearance known by causing a tail of growth on the main pyridoxal zone as observed experimentally.

Examination of the replicate chromatograms 1 and 2 (Fig. 2) of the more dilute sample again reveals 2 zones of growth, one due to a new substance moving more slowly than pyridoxamine, and the other moving like pyridoxal itself. There is little trace seen of the band of growth connecting the zones as observed in analyzing the more concentrated sample. This result suggests the formation of a second more stable derivative of pyridoxal and cysteine whose existence is not so readily disturbed by dilution and which moves at about the same rate as the unstable unknown substance whose existence was noted in the more concentrated sample. As a consequence, for the more concentrated sample only one composite zone is seen at the top of the chromatogram and may be due as indicated above to two substances.

The reaction of pyridoxal and cysteine may take place in a manner similar to that of other simple aldehydes and aldoses with cysteine as demonstrated by Schubert.⁸ Thus a simple adduct may form between the cysteine sulfhydryl group and pyridoxal. This reaction might be expected to be quite reversible. The adduct may then cyclize to give a thiazolidine ring derivative of vitamin B₆. The latter compound would probably be more stable to hydrolysis than the 1st adduct mentioned. This compound might represent the slow moving substance observed on chromatographing the more dilute sample.

The compounds formed from pyridoxal and cysteine for which evidence has been presented above, may represent new forms of vitamin B₆ of physiological interest hitherto unrecognized because of their unstable character.

It might have been quite difficult to demonstrate such a reaction between cysteine and pyridoxal with the usual form of microbiological assay.

A further result of practical interest is the finding that many preparations of synthetic nature such as pyridoxal itself, and pyridoxal phosphate are impure and contain foreign substances which may themselves be active as growth substances replacing B₆. Thus the present technique provides a delicate test of purity for standard preparations of growth factors used in biological and biochemical investigations.

Studies are now being conducted on natural products using this method. These will be reported at a later date.

The potential usefulness of the general technique involving a combination of paper partition chromatography and microbiological indicators should be of value in studies on the differentiation of other growth factors such as folic acid which occur in nature in more than one chemically defined form. This consideration also prompted the present report.

Summary. 1. A new technique of resolving and identifying the members of the B₆ family of growth factors is described. It involves the use of paper partition chromatography coupled with a microbial indicator.

2. Using this method it has been demonstrated that pyridoxal undergoes a non-enzymatic transamination reaction even in the cold. Pyridoxal also appears to react reversibly with other amino acids.

3. The reaction between cysteine and pyridoxal differs from that with other amino acids in that one can demonstrate the formation of at least one adduct which moves more slowly on the chromatogram than does pyridoxamine. This reaction may produce a new form of vitamin B₆ which may also have a transient existence in nature.

4. The technique described should prove of value as a means of establishing the purity of the various forms of vitamin B₆ used in biological and biochemical studies.

5. The general technique employing paper partition chromatography and microbial indicators should be of value in resolving other multiform vitamins into their constituent parts.

⁸ Schubert, M., *J. Biol. Chem.*, 1939, 139, 601.

Influence of Hemorrhage and of Albumin Injections upon the Sodium Concentration of Human Plasma.*†

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The purpose of this paper is to present data on the change in concentration of plasma sodium following phlebotomy in 4 normal subjects as well as on the changes in plasma sodium concentration after injections of salt-poor human albumin and/or concentrated plasma in several patients with malnutrition and hypoalbuminemia. The latter were part of a larger group in which other effects of administering salt-poor albumin and concentrated plasma were studied and reported.¹

Methods. Control blood samples were drawn without stasis from the antecubital vein with the subject in the supine position. All succeeding specimens were obtained from the opposite arm. Clotting was prevented by moistening the barrel of the syringe with a small amount of a commercial heparin solution. After thorough mixing the samples were transferred to hematocrit tubes 8 mm in diameter and centrifuged at 3,000 r.p.m. for 40 minutes, after which the supernatant plasma was carefully removed. One cc of the plasma thus obtained was diluted to 100 cc and thoroughly mixed. A Perkin-Elmer flame photometer was used for making the sodium determinations. A standard solution for calibration contained 50 parts of sodium, as sodium chloride, per million (50 mg per liter) in distilled water. This solution read 100 on the machine. Intermediate values were obtained with standard solutions of concentrations varying from 0 to 50 mg per liter. The calibration curve deviated only slightly from a straight line. Readings of the unknown

samples were recorded and corresponding sodium concentrations were determined from the curve. Frequent resettings of the zero and standard were made while performing each determination. Five readings were recorded for each sample and the average used as the most accurate value. The maximum range noted in the 5 readings was about 1.5 scale divisions, the average deviation ± 1 division, equivalent to approximately ± 1 milliequivalent of sodium. The flame photometer determinations on 3 samples of plasma were checked by gravimetric methods. Agreement within 2 milliequivalents was obtained. For a complete discussion of use and limitations of the flame photometer for such purpose the reader is referred to the paper of Held.²

Findings. Sodium concentrations, determined in 7 normal males 23 to 49 years of age, at intervals of 5, 10, 15, 20, 60, 120, and 180 minutes, showed little variation. The concentrations ranged from 143 to 157 milliequivalents per liter in the several subjects. The extreme differences noted in the 2 to 6 successive samples drawn from the same subject were only 1 to 5 milliequivalents per liter. The average normal value of 147 milliequivalents per liter is in close agreement with the figure of 144 milliequivalents per liter reported by Marinis, Muirhead, Jones and Hill.³

Effect of Phlebotomy. In 4 normal young men the plasma sodium concentration was measured before, immediately after and at intervals of $\frac{1}{2}$, 1, 2, 3, and 24 hours after rapid removal of 500 cc of blood. The sodium concentration fell significantly in each instance. These data are recorded in Fig. 1. In one subject the plasma potassium was simul-

* The serum albumin used in this study was processed by the American National Red Cross from blood which it collected from volunteers.

† Aided by a grant from the Commonwealth Fund.

1 Elman, R., Kelly, F. J., and Simonsen, D. H. in press.

2 Held, P. M., *J. Biol. Chem.*, 1947, **167**, 499.

3 Marinis, T. P., Muirhead, E. E., Jones, F., and Hill, J. M., *J. Lab. and Clin. Med.*, 1947, **32**, 1208.

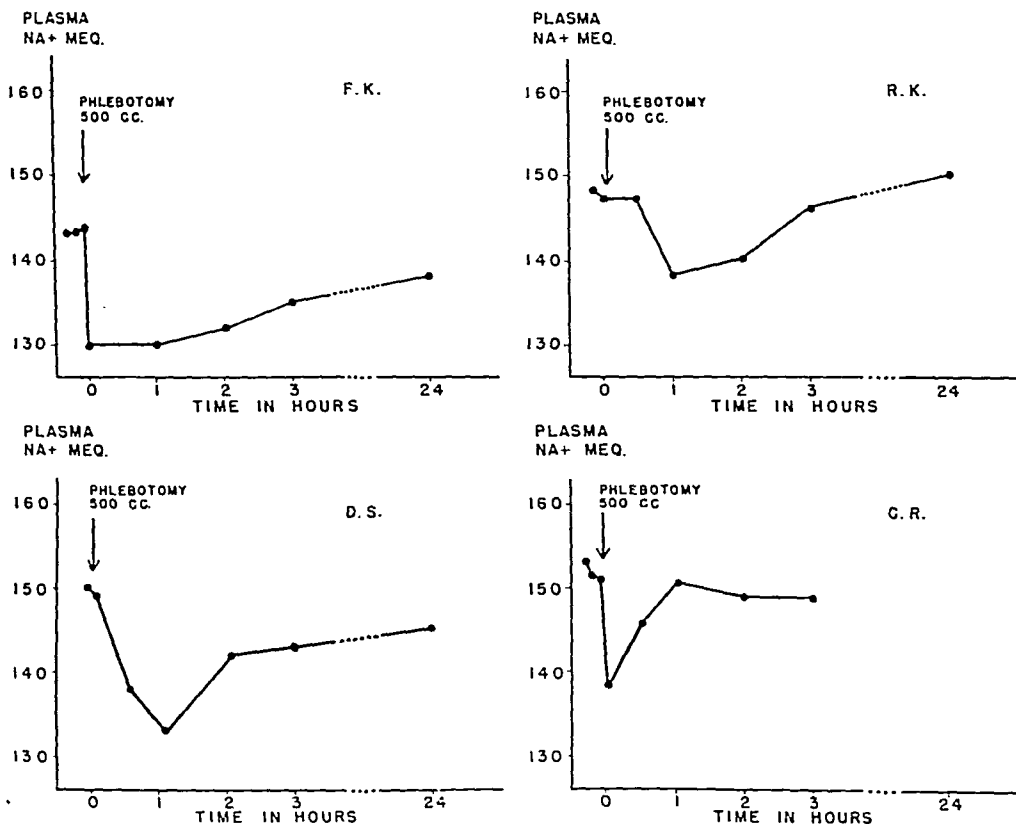


FIG. 1.

Fall in Serum Sodium Following Phlebotomy.

The 4 curves shown above represent observations on 4 healthy donors bled 500 cc. Note the constant drop in the plasma sodium concentration with its return to normal varying from 1 to 3 or more hours.

taneously determined, but no change was noted.

Effect of Injection of Albumin and of Plasma. In 5 patients having malnutrition, hypoalbuminemia and edema, the plasma sodium concentration was measured before, immediately after, and at intervals of 1, 3, 6, and 24 hours after the infusion of either 25% salt-poor human albumin or concentrated plasma.† (Table I) Four patients were given 7 injections of albumin; 1 patient received albumin on one occasion and concentrated

plasma 2 days later; one patient received concentrated plasma alone. Following 5 of the 7 albumin injections, the plasma sodium concentration fell significantly, the maximum decrease occurring immediately after infusion (at which time the plasma volume showed its maximum increase).² In one case (B.P., Table I) in which plasma potassium concentration was simultaneously determined, no significant change was found. In the 2 patients receiving concentrated plasma the plasma sodium content increased significantly, the highest value being obtained 3 hours after injection in both. Immediately after plasma infusion the plasma volume was increased maximally, as with albumin, and at this time the plasma sodium concentration had changed little, or not at all.

† Two 500 cc units of Red Cross lyophilized plasma were dissolved in 1 unit (500 cc) of distilled water. The sodium content was determined by us to be 3.80 g per 500 cc; that of the 25% albumin solution was recorded by the manufacturer as 0.3 g per 100 cc.

TABLE I.
Plasma Sodium Concentration (Milli-equivalents per liter) Before and at Various Intervals Following the Injection of Salt-poor Human Albumin and Double Strength Plasma in Patients with Malnutrition.

Subject	Age	Sex	Diagnosis	Injection	Plasma Na ⁺ concentration (meq.)					Max. decrease (meq)
					Before	After	1 hr	3 hr	6 hr	24 hr
B.E.	71	M	Carcinoma head of pancreas	Albumin (50 g)	150	138	150	156	147	—
G.H.	45	F	Non-tropical sprue	" "	150	149	148	149	142	—12
B.P.	48	M	Cirrhosis	" "	137	133				—8
E.R.	75	M	Carcinoma of pancreas	" "	144	130				—14
				" "	136	137	137	141		—14
				" "	143	130	146			—13
				" "	149	139.5	139.5	141.5	143.5	—9.5
G.S.	71	M	Cirrhosis	Plasma (470 cc double strength)	141	140	141	148	139	141
				Plasma (285 cc double strength)	138	142	141	148		138

Discussion. The fall we observed in the plasma sodium concentration following either hemorrhage or the injection of 25% salt-poor human albumin solution is assumed to be a part of the hemodilution process and thus resolves itself ultimately to a discussion of the source of the fluid that enters the circulation following such procedures.

In a review of the literature we have found but one report on the changes in serum sodium and potassium following hemorrhage. Stewart and Rourke⁶ reported that in 4 normal dogs bled 2 to 3.5% of their body weight, samples drawn 12 hours after the hemorrhage showed a fall in serum sodium, potassium and protein concentration, and variable concentrations of the chloride, carbon dioxide and non-protein nitrogen. These changes occurred whether the animals were allowed fluids by mouth or not. Our subjects were given neither food nor fluids by mouth during the first hour. They remained in bed until the ½ hour samples had been drawn and were then permitted to be up and about. After the one-hour sample was drawn food and fluids were permitted *ad libitum*. The data presented herein in normal human subjects confirm Stewart and Rourke's findings in dogs.

Ashworth, Muirhead and Hill⁴ studied the effect of concentrated citrated and defibrinated dog and human plasma on the serum sodium and potassium concentration of normal dogs. They found no change in the samples drawn 15, 20, 60 minutes and 4 hours after injection. Since the plasma volume (dye method) as well as the extracellular fluids (thiocyanate method) increased after infusion, without change in serum sodium concentration, these authors concluded that the fluid added to the circulation was derived from the intracellular space. We observed an increase in the serum sodium concentration 3 hours after the injection of concentrated citrated plasma in 2 patients.

Heyl, Gibson and Janeway⁵ in their study of the effect of concentrated solutions of

⁴ Ashworth, C. T., Muirhead, E. E., and Hill, J. M., *Am. J. Physiol.*, 1942, **136**, 194.

⁵ Heyl, J. T., Gibson, J. G., 2nd, and Janeway, C. A., *J. Clin. Invest.*, 1943, **22**, 713.

human and bovine serum albumin on the blood volume after acute blood loss in normal men, reported no change in the serum or urinary potassium concentration, but their albumin solutions were not salt-poor. In 5 of the 7 patients in whom we injected salt-poor albumin a significant fall in plasma sodium occurred. In the single patient in whom potassium was measured, no change was noted. It should be emphasized that the fall in sodium concentration was most marked immediately after infusion, at which time the plasma volume had increased by a volume more than 3 times that of the fluid injected. The magnitude of the fall in sodium concentration was much less than could be accounted for by hemodilution with a sodium-free solution, hence the fluids responsible for the dilution probably contained sodium but in lower concentration than in plasma.

Because intracellular fluid is poor in sodium and rich in potassium, it is logical to assume that much of the diluting fluid must have come from this source, in which case one might expect a rise in plasma potassium. The latter was measured in 2 cases, but no change

was found. However, renal clearance of potassium is so rapid that this discrepancy may not be significant. Indeed, Stewart and Rourke,⁶ in the experiments cited earlier, found an increase in potassium output in the urine after hemorrhage. The red cells themselves might be assumed to be the source of the diluting fluid, but this could be true only if a rapid two-way shift of fluid were involved, inasmuch as no change in the size of red cells was observed after the injection of albumin.²

Summary. 1. Following phlebotomy in each of 4 normal male subjects, there was a transient fall in the plasma sodium concentration.

2. Following injections in 4 patients of a 25% solution of salt-poor human albumin solution, a similar fall in the plasma sodium concentration was noted.

3. It is concluded that sodium-poor fluid is added to the plasma, presumably from the intracellular space, following hemorrhage, as well as following injection of salt-poor human albumin.

⁶ Stewart, J. D., and Rourke, G. M., *J. Clin. Invest.*, 1936, **15**, 697.

16360

Environmental Conditions Which Initiate Sweating in Resting Man.

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During the course of studies^{1,2,3} on the influence of environmental conditions upon the rate of water loss from the skin of man,

* Aided by grants from the Life Insurance Medical Research Fund, a War Contract No. WD-49-007-MD-389, Helix Institute for Medical Research, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

¹ Burch, George, and Winsor, Travis, *J. Clin. Invest.*, 1944, **23**, 937.

² Winsor, Travis, and Burch, George E., *Arch. Int. Med.*, 1944, **74**, 428.

³ Burch, George E., and Winsor, Travis, *Arch. Int. Med.*, 1944, **74**, 437.

observations were made to learn the environmental temperature and relative humidity which would initiate sweating in normal man.

Eleven subjects of both sexes, varying in ages from 12 to 59 years, were permitted to rest quietly on a hospital type of bed in an air-conditioned room. They were clothed only in a cotton gown. The rate of water loss from the skin of the epigastrium and volar surface of the right forearm was determined by a method previously described.⁴

⁴ Burch, George E., and Sodeman, William A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 190.

TABLE I. Results of Studies to Learn Environmental Conditions Necessary to Initiate Sweating in Normal Man Resting in a Hospital Type Bed.*

Subject	Age	Sex	Color	Before sweating			Sweating		
				Temp. (C)	Relative humidity (%)	Rate of water loss mg/cm ² /10 min. Epigastrium Forcarm	Temp. (C)	Relative humidity (%)	Rate of water loss mg/cm ² /10 min. Epigastrium Forcarm
1	22	F	G	35	85	6.0	37.8	60	19.8
2	50	F	W	32.2	54	7.1	34.4	50	8.7
3	25	F	W	34.4	28	7.4	36.7	26	13.3
4	40	M	W	30.0	62	5.8	33.8	58	7.3
5	12	F	G	32.2	90	4.5	34.4	70	7.5
6	31	F	G	32.2	80	5.6	35.5	56	11.6
7	49	F	G	31.1	42	5.2	33.3	42	20.0
8	34	M	G	36.1	26	4.6	38.9	28	7.5
9	19	M	G	38.9	9.5	5.5	38.9	9	6.9
10	17	M	W	32.3	22	7.0	35.5	20	11.4
11	59	M	W	33.3	26	4.0	34.4	26	5.9
Mean				33.5	47.9	5.7	35.8	40.5	10.9
Max.				38.9	90	7.4	38.9	70	20.0
Min.				30.0	9.5	4.0	33.3	9	5.9

* The water lost before sweating is lost by diffusion. The onset of sweating in subjects Nos. 5 and 6 showed local variations.

After the subjects had rested for at least 60 minutes under comfortable environmental conditions (temperature and relative humidity approximately 20.4°C and 50% respectively) the room temperature was elevated 3°C at a time, and the relative humidity was changed in a variable fashion. After the subjects had been exposed for 15 minutes to the new room conditions their rate of water loss was measured. This procedure was continued until an atmospheric condition was reached which was associated with a definite increase in rate of water loss. Occasionally, as a check, the room temperature or humidity or both were lowered, the rate of water loss was measured, the temperature or humidity or both were raised again, and another water collection was made.

Results are summarized in Table I. Sweating was initiated by a temperature of about 36°C when the relative humidity was about 40%; with a low relative humidity, a higher air temperature was required. Sweating began at a temperature of 39°C, even though the relative humidity was as low as 9%. In one patient sweating was cyclic, *i.e.*, it alternately occurred and ceased for 15-minute periods, even though the room conditions remained unchanged. These observations suggested that sweating cooled the subject sufficiently to abolish the need for this mechanism for a certain length of time. He accumulated heat, sweated again, cooled his body, and then stopped sweating. This cyclic sweating conserves water and electrolytes.

Such observations indicate that environmental temperature of 34.4°C and relative humidity of about 50% are essentially the threshold level for sweating in normal man resting in bed. Increased exercise and rate of heat production are accompanied by a proportionate lowering of the threshold.

When the studies were prolonged, subjects became restless and irritable, and "nervous" or "psychogenic" sweating resulted, which made it impossible to observe the thermal sweating.

Summary. Observations were made on 11 subjects of varying ages to discover the environmental conditions necessary to initiate

sweating in man. These studies, performed in a room designed for close control of temperature and humidity, indicate that an environmental temperature of 34.4°C and relative humidity of about 50% are essentially the

threshold level for sweating in normal man resting in bed. Exercise and increased rate of heat production are associated with a proportionate lowering of the threshold.

16361

Utilization of Glutamic Acid in the Presence of High Levels of Pteroylglutamic Acid.

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It has been recently suggested that pteroylglutamic acid (PGA) may function as a metabolic antagonist for glutamic acid.^{1,2} The statement was made that "folic acid may competitively interfere with the nutrition of the spinal cord just as certain vitamin deficiencies in experimental animals may be caused by closely related chemicals. Thiamine deficiency, for example, may be induced by the administration of pyrithiamine, and pantothenic acid deficiency by pantooyltaurine." It was further suggested that PGA could interfere with the metabolism of the central nervous system *in vivo* or *in vitro*.¹

The analogy between folic acid and such "anti-vitamin" compounds as pyrithiamine appears to be inappropriate. Folic acid is a member of the vitamin B complex, and is itself inhibited by "anti-vitamin" compounds which are comparable to pyrithiamine and

pantooyltaurine. These compounds include "methyl folic acid,"^{3,4} pteroylaspartic acid,⁵ 4-amino pteroylglutamic acid,⁶ certain pteridines,⁷ a sulfonyl-substituted benzimidazole analogue of PGA,⁸ and N¹⁰-methyl pterioic acid.⁹

Since representative species of bacteria require both folic acid and glutamic acid, it is obvious that folic acid does not competitively interfere with the metabolism of glutamic acid by these bacteria. However, because the response to glutamic acid obtained with *L. casei* and *S. faecalis* R is so quantitative and sensitive, an experiment was made to determine whether massive amounts of PGA would slow up growth on suboptimal levels of glutamic acid. A culture medium deficient in glutamic acid¹⁰ was used and PGA was added at levels of 0.01 µg, 1 µg, 10 µg, to 3 series of tubes containing varying amounts of glutamic acid. The results are illustrated in Fig. 1.

The growth curves indicate that increasing the level of PGA 1000-fold had no inhibitory

¹ Anonymous, *New England J. Med.*, Editorial, 1947, 237, 713.

² Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, 3, 68.

³ Martin, G. J., Tolman, L., and Moss, J., *Arch. Biochem.*, 1947, 12, 318.

⁴ Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, 169, 427; Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, 65, 368.

⁵ Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, 170, 323.

⁶ Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, 69, 2567.

⁷ Daniel, L. J., Norris, L. C., Scott, M. L., and Heuser, G. F., *J. Biol. Chem.*, 1947, 169, 639.

⁸ Edwards, P. C., Starling, D., Mattocks, A. M., and Skipper, H. E., *Science*, 1948, 107, 119.

⁹ Smith, J. M., Jr., and Cosulich, D. B., *J. Am. Chem. Soc.*, in press.

¹⁰ Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, 1944, 155, 591.

ml. 0.05 N NaOH
(*L. casei*)

galvanometer
reading
(*S. faecalis* R)

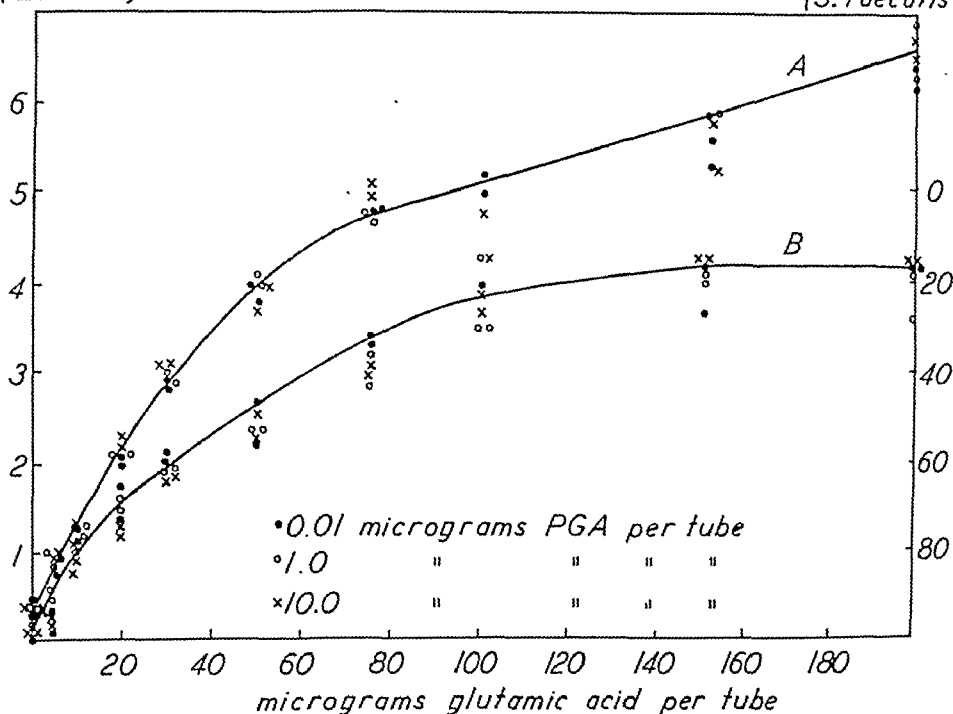


FIG. 1.

Growth response of *Lactobacillus casei* (curve A) and *Streptococcus faecalis* R (curve B) at 72 hours to glutamic acid in the presence of various levels of pteroylglutamic acid (PGA).

effect upon the utilization of glutamic acid by either *L. casei* or *S. faecalis* R. The scattering around the experimental points was within the anticipated limits of the assay method and in no case was the trend dependent upon the level of PGA. The experiment also demonstrates that neither organism is able to use PGA as a substitute for glutamic acid.

In view of the suggestion that PGA interferes with the metabolism of glutamic acid by the central nervous system *in vitro*, further experimental work was done to determine the effect of PGA on the utilization of glutamic acid by brain and kidney slices. The slices were suspended in Ringer-phosphate solution and the rate of oxygen consumption was determined at 37° in the Warburg-Barcroft apparatus. The minimum concentration of glutamic acid ($3 \times 10^{-3}M$) which caused an appreciable stimulation of oxygen consump-

tion, was used, for this concentration would presumably give the most favorable conditions for observing any competitive interference with the utilization of this acid.

The results are given in Table I. Q_{O_2} values (cu mm of oxygen consumed per mg dry tissue per hour) are included for brain slices for both the first hour and the first 3 hours of respiration, for the consumption of oxygen was not a linear function with time. The results can be evaluated by examining the values obtained for increases in Q_{O_2} caused by adding glutamic acid to the medium as summarized in the last 2 columns of Table I. When the concentrations of glutamic acid and PGA were equimolar there was no significant depression in the utilization of glutamic acid by brain or kidney slices. Had the PGA interfered with the utilization of glutamic acid, the addition of PGA should have

TABLE I.
Effect of Addition of Pteroylglutamic Acid to the Medium on Utilization of Glutamic Acid by Surviving Brain and Kidney Tissue Slices of Normal Rats.

Q _{O₂}												
Exp.	PGA conc.	Brain slices									Increase in Q _{O₂} with addition of glutamic acid*	
		1st hr			3-hr interval			Kidney slices				
		(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)		
		Brain	Kidney									
1	0	3.4	4.6	6.1	1.9	3.4	4.7	10.4	12.8	13.3	2.8	2.9
	3 × 10 ⁻⁴ M	3.4	5.8	6.3	1.9	4.2	5.0	9.2	11.8	12.0	3.1	2.8
2	0	5.0	6.8	9.6	3.3	5.2	7.4	11.8	19.2	21.9	4.1	
	3 × 10 ⁻⁴ M	4.4	8.2	9.8	3.0	6.1	7.6	11.6	19.6	—	4.6	
3	0	4.6	6.0	7.2	2.8	4.3	5.4	9.9	12.0	13.1	2.6	3.2
	3 × 10 ⁻³ M	3.6	6.0	6.8	2.2	4.2	5.2	9.0	11.6	12.7	3.0	3.7
4	0	5.0	8.6	7.3	3.3	6.4	6.0	15.0	15.2	18.0	2.7	3.0
	3 × 10 ⁻³ M	4.4	7.2	7.9	2.9	5.3	6.0	12.2	15.2	14.1	3.1	1.9

(a) No glutamic acid added to medium.

(b) Medium contained 3 × 10⁻³ M added glutamic acid.

(c) Medium contained 10⁻² M added glutamic acid.

*The values are calculated from the results with 10⁻² M glutamic acid. The values for brain slices were determined from the 3-hour respiration interval.

reduced the magnitude of the Q_{O₂} increases, but no such reduction was observed. The maximum PGA level which has been reported in the blood of human subjects is 0.8 μg per ml¹¹ following the intravenous administration of 15 mg of PGA. In the present study levels as high as 1323 μg PGA per ml of medium were without significant effect on glutamic acid metabolism.

In another series of similar experiments, the animals were either made severely deficient in PGA or fed a high level of PGA for a prolonged period. The birds on the deficient diet developed a severe cervical paralysis,^{12,13} and the deficient rats showed a mild cytopenia. The animals were sacrificed when the marked signs of the characteristic deficiency developed. Control animals were fed purified diets^{4,12} containing adequate levels of PGA (1 mg PGA per kg diet for rats and 2 mg per kg of diet for turkeys). Because of the non-linear oxygen consumption curves obtained

with brain tissue the control animals were sacrificed simultaneously with the experimental animals and the tissue slices were prepared from both at the same time. The respiration of the tissue slices was measured as described above, except that no PGA was added to the medium. No difference between the behavior of the tissues from the deficient and control animals was found (Table II).

The effects of feeding large amounts of PGA and of PGA deficiency in the rat on glutamic acid metabolism by brain and kidney tissue *in vitro* were compared (Table II). The "high-PGA" diets contained 100 mg PGA per kg and were fed for several weeks. The tissues from two series of animals showed no significant differences in the tests.

Discussion. In studies with various species of animals PGA, like the other B-complex vitamins, has been shown to be a substance of very low toxicity.¹⁴ Prolonged dosage of human subjects with 50 mg of PGA daily has been reported not to produce adverse symptomatology.¹⁵ Recent statements² have

¹¹ Denko, C. W., Abstracts of Papers, American Chemical Society, 112th meeting, New York, 25 C, 1947.

¹² Jukes, T. H., Stokstad, E. L. R., and Belt, M., *J. Nutrition*, 1947, **33**, 1.

¹³ Richardson, L. R., Hogan, A. G., and Kempster, H. L., *J. Nutrition*, 1945, **30**, 151.

¹⁴ Harned, B. K., Cunningham, R. D., Smith, H. D., and Clark, M. C., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 289.

¹⁵ Berry, L. J., and Spies, T. D., *Blood*, 1946, **1**, 271.

dietary supplement of PGA. Brain and kidney tissue slices from rats receiving a high dietary level of PGA respired at the same rate as corresponding tissues from control rats, with or without added glutamic acid.

ADDENDUM: After this manuscript was submitted, an article appeared (Grossowicz, N., *J. Biol. Chem.*, 1948, **173**, 729) which reported that

glutamine inhibited the growth-promoting effect of glutamic acid for *Staphylococcus aureus*, and that this inhibitory effect of glutamine could be overcome by pteroylglutamic acid, by glutathione, or by extra glutamic acid. These results apparently indicate that under certain circumstances pteroylglutamic acid and glutamic acid may function interchangeably in the nutrition of micro-organisms.

16362 P

Effect of Tween 80 on Certain Strains of *C. diphtheriae*.

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In attempts to prepare a fluid synthetic medium for general use in diphtheriology, difficulty was encountered in that recently discovered minimus strains^{1,2,3} and a few, closely related, minute-colony strains, failed to grow in a medium suitable for most other strains. The synthetic medium used consists of amino acids, inorganic salts, carbohydrates and growth factors in as pure form as could be obtained. It has no peptone, casein or other constituents of variable or unknown composition. Details of the medium will be published elsewhere.

The minimus strains have also uniformly failed to grow well in infusion broth. The granular nature of growth of the minimus strains in infusion broth suggested the possibility that a surface tension reducent might facilitate growth, following the line of thought suggested by Dubos *et al.*^{4,5,6} in their studies of the tubercle bacillus. Accordingly, Tween

80 was added aseptically from a freshly prepared, sterile, aqueous solution, in a concentration of about 0.05%, to the synthetic medium just before inoculation. Immediate and striking stimulation of growth of the minimus-type strains was observed. Concentrations of Tween up to about 0.5% have since been used with no apparent inhibition of the minimus type. No optimum or inhibitory range has yet been determined.

With other types of diphtheria bacilli, the results of adding Tween 80 to the medium are somewhat variable. Some strains, particularly of the mitis type, appear slightly inhibited; others show little or no difference in growth response to Tween. Gravis and gravis-like strains which normally grow with pellicle formation, lose their pellicle and grow diffusely throughout the medium. No types other than minimus are stimulated to any great degree, with the exception of a few minute-colony forms sent by Dr. McLeod from Leeds sometime ago. These strains were designated by McLeod as the intermedius type. It is of interest that the 4 strains of this group which Frobisher found to be identical with minimus type³ were more markedly stimulated by addition of Tween than were the other 6.

Similar stimulation of growth of the mini-

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¹ Eller, C. H., and Frobisher, M., Jr., *Am. J. Hygiene*, 1945, **42**, 179.

² Frobisher, M., Jr., *et al.*, *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 330.

³ Frobisher, M., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 304.

⁴ Dubos, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 361.

⁵ Dubos, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 56.

⁶ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

mus-type strains was observed when Tween 80 was added to tubes of heart-infusion broth. The difference in growth resulting from addition of Tween 80 to this medium was not so striking as in the synthetic medium, since growth of the minimus strains is normally better in heart-infusion than in synthetic medium. Again, the results with strains of other types were variable. None, other than minimus type, was definitely stimulated, with the exception of the minute-colony forms noted above.

Plates of McLeod's chocolate agar were prepared; half with about 0.3% Tween, and half without. A small loopful of 24-hour broth culture of various types of diphtheria organisms was streaked on both media. Results of this experiment paralleled those obtained with liquid media. Minimus colonies were markedly increased in size, from less than 0.1 mm diameter on plates without Tween to diameters of 2.0 to 3.0 mm on the plates with Tween added. Colony size of other types was variable, the intermedium strains of McLeod being the only ones which showed any significant increase in diameter (0.1 mm to 1.5 mm).

It has been repeatedly observed in this laboratory⁷ that at least 1 ml of 48-hour culture of the minimus type organisms is required to kill guinea pigs and that even that dose kills irregularly; whereas, the lethal dose of the gravis and mitis types approximates 0.2 ml or less. Though the minimus strains grow more sparsely than do the other types, after seeing the broth cultures it is a little difficult to accept the idea that this difference in lethal dose is due to *numbers* of organisms alone. Therefore, 48-hour broth cultures of a minimus strain, grown with 0.05% Tween, and in the same medium without Tween, were injected intradermally into duplicate guinea pigs in amounts of 0.2, 0.5, and 1.0 ml. All animals receiving the Tween culture were dead within 72 hours. Autopsy showed typical signs of diphtheritic deaths. Only one pig with the culture without Tween died, and that was one of the two which had

received 1.0 ml of culture. All other experimental and control animals survived without evidence of disease for more than two weeks. This experiment had been repeated in part with a second minimus strain, with the same results.

In alcohol-ether and acetone fractionation of the gravis, mitis and minimus types, Parsons⁸ found that the minimus strains contained more lipid material than did gravis or mitis strains. This suggests a relationship of wetting agent (Tween 80) to the lipid-rich minimus type, analogous to the relationship of the same wetting agent to the lipid-rich tubercle bacilli, shown to exist by Dubos *et al.* The surface tension of the synthetic medium was found to be relatively high. Tween 80 reduces this surface tension markedly. That the surface tension of the heart-infusion without Tween is considerably lower than that of the synthetic medium might partially account for the normally better growth of the minimus strains in heart-infusion broth.

The role of Tween as a non-toxic source of oleic acid must also be considered in view of the work of Cohen *et al.*,⁹ who found oleic acid to be one of two growth factors, present in serum and milk, essential for a gravis strain when very minute inocula were used on solid medium. No statement was made by these authors as to the effect of these factors in liquid media.

Summary. It has been observed that the addition of Tween 80 to solid and liquid media markedly stimulates the growth of minimus type diphtheria bacilli. This suggests a relationship between this wetting agent and the lipid-rich minimus organisms analogous to the relationship of the same agent to the lipid-rich tubercle bacilli, demonstrated by Dubos *et al.* There is also evidence that Tween 80 enhances the virulence of the minimus strains.

The writer is indebted to Dr. Martin Frobisher, Jr., for his stimulating interest and help and Dr. Elizabeth I. Parsons for her permission to cite unpublished data.

⁸ Parsons, E. I., unpublished data.

⁹ Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, 1941, **41**, 581.

⁷ Frobisher, M., Jr., and Parsons, E. I., unpublished data.

16363

Hyaluronidase Content of Normal and Inflamed Guinea Pig Skin.

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In the presence of hyaluronidase the epidermal challenge reaction in guinea pigs sensitized to paraphenylenediamine is markedly increased as compared to control animals (Mayer and Kull¹). This observation suggested that hyaluronidase plays a role not only in the spread of invasive organisms and the ensuing bacterial inflammation (Duran-Reynals²), but also in certain non-bacterial inflammations of the skin.

In order to obtain further evidence of this hypothesis it was desirable to determine whether the hyaluronidase content of the guinea pig skin was increased during allergic and non-allergic inflammations.

Methods. Guinea pigs were sensitized to paraphenylenediamine in the manner described by one of us.³ After sensitization had been established, the animals were challenged with 10% paraphenylenediamine in petrola-

tum applied to the left flank, and sacrificed 24 hours later. 3 x 4 inch strips of skin were removed from challenged and unchallenged sites.

Physical inflammations were produced by application of heat (water at 70°C) for 1 and 2 minutes respectively on the shaven skin and by ultraviolet irradiation (Hanovia lamp, distance 12 inches) for various exposure periods. Skin samples were obtained from both inflamed and normal sites.

Identical weights (based on dry weight) of inflamed and non-inflamed skin were successively subjected to freezing and thawing and then ground with sterile sand. The resulting tissue-brei was extracted with 10 ml of 0.1 M acetate buffer, pH 6.0, containing 0.15 M NaCl, and centrifuged. The supernatant fluid was assayed viscosimetrically according to the method of McClean and Hale,⁴

TABLE I.

Treatment	Guinea pig No.	Intensity of inflammation	Hyaluronidase content skin-viscosity reduction units per g dry wt*	
			Normal skin	Inflamed skin
Paraphenylenediamine dermatitis	1	++++	.015	.456
	2	++++	.043	.185
	3	++++	.157	.925
	4	++++	.022	.856
	5	++++	.045	1.110
Heat-induced inflammation	6	++++	.00	.391
	7	+++++	.034	.556
Ultraviolet erythema	8 (2')	+	.040	.0061
	9 (5')	+	.00	.054
	10 (20')	++	.0126	.034
	11 (30')	++	.045	.0

* The skin viscosity reduction unit was obtained by applying the formula of McClean and Hale⁴ with the modification of 60' being equivalent to one unit. The values are extrapolated by applying $\sqrt{\text{time}}$ vs. viscosity.

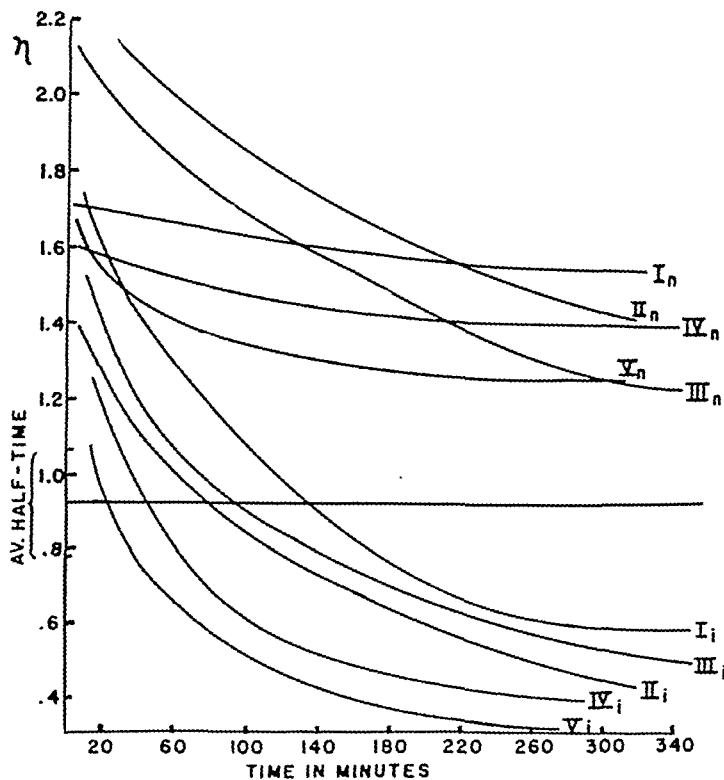
† Minutes of exposure to ultraviolet. One minute equal to one guinea pig skin erythema dose.

¹ Mayer, R. L., and Kull, F. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 392.

² Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

³ Mayer, R. L., *Arch. f. Dermat. u. Syph.*, 1931, **163**, 223.

⁴ McClean, D., and Hale, C. W., *Biochem. J.*, 1941, **35**, 159.



GRAPH 1.
Viscosity Reduction with Normal and Inflamed Skin from Guinea Pigs
Allergic to Paraphenylenediamine.

I_n , II_n , III_n , IV_n , and V_n are the viscosity reduction curves obtained from normal skin; I_i , II_i , III_i , IV_i , and V_i are the viscosity reduction curves obtained from inflamed skin.

using 0.4 cc of the above supernate and 0.6 ml of 0.417% hyaluronate (prepared from umbilical cords according to Kass and Seastone⁵) dissolved in the acetate buffer.

Results. The results of the experiments on 11 animals shown in Table I indicate that the free hyaluronidase content in allergic or heat-inflamed skin is up to 40 times as high as that of the corresponding normal skin.

Unlike the inflammations induced by the allergic challenge or by heat, the 24-hour ultraviolet reaction after the administration of 2 to 60 guinea pig erythema doses did not result in a consistent increase of free hyaluronidase. No increases were observed in 2 skins which had been irradiated for 2- and 30-minute periods respectively, while 2 other skins, irradiated for 5- and 20-minute

periods respectively showed a slight but not significant increase.

Negative results were also obtained with 2 guinea pigs sensitized to horse serum upon subjection to local Arthus reactions.

Graph 1 shows the course of viscosity reduction of the tests in animals presenting allergic inflammation (animals 1-5).

Discussion. While hyaluronidase in bound form is known to exist in the normal skin (Meyer⁶), our experiments have shown that normal guinea pig skin also contains very small amounts of free hyaluronidase. During inflammation produced by a challenging application of paraphenylenediamine on sensitized skin or after treatment of the skin with hot water, the amounts of polysaccharide-depolymerizing enzymes (probably chiefly hyaluronidase) are markedly increased—up

⁵ Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

⁶ Meyer, K. F., *Physiol. Rev.*, 1947, **27**, 335.

to 40 times.

Ultraviolet irradiation, producing definite erythema in guinea pigs, results in an occasional but very slight increase of these enzymes, contrary to the Arthus phenomenon, which in 2 rabbits did not result in increased amounts. It is noteworthy that the ultraviolet treatment and the Arthus phenomenon primarily concern the cutis, whereas the heat-induced inflammation and the epidermal allergic challenge reaction concern mainly the epidermal tissue.

Although the previous experiments on the influence of antihistaminics upon the hyaluronidase reaction¹ suggest a relationship

between skin inflammation and the liberation of hyaluronidase, further experiments are necessary to determine whether hyaluronidase plays an active role during these inflammations or is only a concomitant factor subsequent to an inflammatory process. Another unsolved problem is the source of the liberated enzyme.

Conclusion. The content of polysaccharide-depolymerizing substances (probably chiefly hyaluronidase) during allergic dermatitis (paraphenylenediamine) and heat-induced inflammation on guinea pig skin is markedly increased.

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Effect of Intravenous Cytochrome C on Capacity for Effort Without Pain in Angina of Effort.

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Within the past few years, reports have appeared on the effect of Cytochrome C* on tissue anoxia. From the clinical point of view, Proger and his co-workers have presented evidence that myocardial anoxia can be relieved by intravenous injection of Cytochrome C, as demonstrated by the prevention or reversal of the electrocardiographic changes induced by breathing 10% oxygen.¹ It is their contention that, under such circumstances, Cytochrome C enhances the uptake of oxygen by the myocardium.

Another method of testing the effect of a drug on myocardial anoxia is to determine the capacity for effort without pain in patients who have angina pectoris brought on by effort. Since this method depends on a subjective end-point, it is necessary to use careful controls and a completely "blind" technique, as described by Gold and his co-

workers, in order to eliminate variable psychosomatic effects.² This method has been used by us in an evaluation of the effect of intravenous aminophylline on angina of effort, and it proved to be a satisfactory investigative technique.³

The subjects of the study were all selected from the active attendance of the Cardiac Clinic. All of them had either arteriosclerotic or hypertensive heart disease or a combination of both, and all presented the symptom of chest pain on effort. No patients were included in this study who had spontaneous chest pain unrelated to effort, excitement, heavy meals, or very cold weather. Also excluded were patients who showed any clinical evidence of congestive failure.

The method consisted of determining the subject's capacity for effort without pain by having him walk back and forth over a

* Cytochrome C used in this study was furnished by Wyeth, Inc., Philadelphia, Pa.

¹ Proger, S., and Dekaneas, D., *J. Pediat.*, 1946, 29, 729.

² Gold, H., Kwit, N. T., and Otto, H., *J. A. M. A.*, 1937, 108, 2175.

³ Bakst, H., Kissin, M., Leibowitz, S., and Rinzler, S., *Am. Heart J.*, in press.

standard set of steps until the onset of his usual type of chest pain. The rate of walking was set by the subject himself, and in any subsequent tests on that same subject, he was kept to the same rate. Every test with Cytochrome C was coupled with a control test on the same day, thus eliminating the effect of the spontaneous variations in pain on different days in the same subject. All tests were preceded by a rest period of one hour, and were conducted in the early afternoon, at least 4 hours after the last meal. In a typical experiment, the patient came to the laboratory at 12:30 o'clock without having had any lunch. He rested at least one hour and was then given the first intravenous injection at a rate of one cc per minute (total of 5 cc per injection). This was followed by a 5 minute rest period, and then the first trial was carried out. As soon as the endpoint was reached, the subject sat down and rested for another hour. He was then given the second injection in the same manner, followed by a 5 minute interval, and then the second trial was conducted.

Cytochrome C was given intravenously in doses of 50 mg in 5 cc of solution. The control material used was physiological saline solution, and because Cytochrome C is red, it was necessary to prevent the patient from seeing what was being injected, lest he have a different psychological effect from a red medicine than from a white one. For this reason, a cloth screen was interposed between the patient and the investigator giving the injections, and the subject's arm was extended through a slit in the screen. The injections were all given by one person, and the observations on capacity for effort were made by the other investigator, who was unaware of which substance had been administered. The sequence of injections was varied so that in some instances the control test preceded the test with Cytochrome C, and in other instances the order was reversed. There was no indication that the subjects of the study were aware that different materials were being injected. There were no consistent reactions to the injection of Cytochrome C which would enable the subjects to detect that they were

receiving a material different from the control solution.

Results. There were 12 pairs of tests conducted on 8 patients. In 4 of the pairs, the trial with Cytochrome C preceded the control trial; in the remaining 8 pairs, the control trial was conducted first. Analysis of the results reveals that Cytochrome C produced no increase in capacity for effort without pain. In fact, there was a mean decrease in performance equal to $2.33 \pm \text{S.E. } 1.95$ trips following the injection of Cytochrome C, as compared with the results obtained with the control injections. Expressed in percentages, this is a decrease of $8.7 \pm 7.2\%$. Since the "t" value derived from these figures is only 1.19, it is concluded that there is no statistically significant difference between the results obtained following injection of Cytochrome C and the results following injection of physiological saline. A summary of the results is found in Table I.

Discussion. Cardiac pain as exemplified by angina of effort has been suggested to be due to an inadequate supply of oxygen to the heart to meet its demands.⁴ The stimulus for pain is believed to be an accumulation of acid metabolites produced by the heart muscle under ischemic conditions.⁵ The possibility of enhancing the tissue uptake of oxygen by means of Cytochrome C in instances of myocardial anoxia is then of special interest in patients with angina of effort. Proger and Dekaneas showed that 50 or 60 mg of Cytochrome C injected intravenously was able to revert to normal in as little time as two minutes an abnormal electrocardiogram produced by breathing a 10% oxygen mixture.¹ Rabinowitch, Elliott, and McEachern have shown that Cytochrome C in doses of 50 mg is taken up by tissues or destroyed within a matter of minutes after injection.⁶

We can assume that the dose of 50 mg of Cytochrome C given intravenously is taken

⁴ Keefer, C. S., and Resnik, W. H., *Arch. Int. Med.*, 1928, **41**, 469.

⁵ Katz, L. N., *Am. Heart J.*, 1935, **1**, 322.

⁶ Rabinowitch, R., Elliot, K. A. C., and McEachern, D., *Canad. M. A. J.*, 1948, **58**, 92.

TABLE I.
Clinical Data and Results of Exercise Tolerance Tests.

Subject	Sex	Age	Diagnosis	No. of trips performed	
				1st trial	2nd trial
A.L.	M	55	Arteriosclerosis, hypertension, enlarged heart, dilated aorta. ECG: Prolonged A-V conduction	24 (S)*	27 (C)†
J.G.	M	63	Arteriosclerosis, enlarged heart, coronary sclerosis. ECG: Ventricular premature contractions	12 (S)	11 (C)
J.M.	M	66	Arteriosclerosis, myocardial fibrosis, sclerotic aorta. Myocardial infarction in 1940. Diabetes mellitus	11 (S) 18 (C)	11 (C) 16 (S)
S.K.	F	66	Arteriosclerosis, hypertension, enlarged heart, myocardial fibrosis. ECG: Bundle branch block	16 (S)	17 (C)
S.S.	M	65	Arteriosclerosis, enlarged heart, myocardial fibrosis, dilated aorta. ECG: Myocardial damage	29 (C) 48 (S)	42 (S) 49 (C)
M.W.	M	70	Arteriosclerosis, enlarged heart, dilated aorta, myocardial, fibrosis. ECG: Myocardial damage. Myocardial infarction, 1938	30 (S)	11 (C)
F.P.	F	58	Arteriosclerosis, hypertension, enlarged heart, myocardial fibrosis. ECG: Myocardial damage	22 (S) 38 (C)	22 (C) 36 (S)
M.F.	M	47	Arteriosclerosis, myocardial fibrosis, aortic sclerosis. ECG: Myocardial damage. Myocardial infarction, 1942	26 (S) 39 (C)	22 (C) 39 (S)

* (S) Physiological saline.

† (C) Cytochrome C.

up or utilized by the tissues within 5 minutes. However, we were unable to demonstrate any increase in the capacity for effort without pain in patients with angina of effort follow-

ing intravenous injections of 50 mg of Cytochrome C as compared with placebo injections of physiological saline solution.

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The Delay in the Action of Digitalis Glycoside (Lanatoside C.)*

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Since the studies of Clark¹ were published, it has been believed² that after the adminis-

tration of digitalis glycosides, a latent period occurs before a cardiac effect is produced.

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In our previous studies however, concerning the effect of different concentrations of Lanatoside C upon the embryonic duck heart, we observed that although the above described latent period did exist, nevertheless, its duration appeared to vary inversely with the

¹ Clark, A. J., *Proc. Roy. Soc. Med.*, 1912, 5, 181.

² Gold, H., Cattell, M., Modell, W., Kurt, N. T., and Kramer, M. L., *Proc.*, 1943, 2, 80.

TABLE I.
Relationship Between Concentration of Digitalis Glycoside and Time of Occurrence of "Digitalis Effect" in Embryonic Duck Hearts.

Digitalis glycoside (mg/cc)	Diameter embryonic sinus (Mm)	Hearts (No.)	Onset: "digitalis effect" (Min.)
.00005	30	15	39
.0001	28	19	22
.0005	32	18	15
.001	33	39	7
.002	30	4	4
.005	30	8	3
.01	30	7	1.75
.05	31	5	0.50
.10	30	10	0.25

concentration of glycoside in contact with the heart. In these same studies however some delay in cardiac response was observed even at the greatest concentration of digitalis glycoside employed (0.001 mg per cc). It seemed important then to discover the effect, at higher concentrations of glycoside, upon the described latent period. The embryonic duck heart lent itself admirably for the purpose because of its relative paucity of tissue which allowed a possible opportunity for changing rapidly, the milieu of the cardiac cells.

Methods. The duck hearts were obtained as previously described^{3,4} from embryos having a vascular sinus of 25-35 mm in diameter. The temperature of the fluid bathing the embryonic heart, was maintained at 35° C which caused more rapid beating than observed in previous studies. Consequently the appearance of A-V block or missing beats also occurred earlier. Either of these two latter abnormalities in rhythm were considered indicators of a "digitalis effect." Lanatoside C, varying in concentration from 0.00005 to 0.10 mg per cc of

Tyrode's solution was employed in all experiments.

Results. As Table I indicates, the delay in "digitalis effect" varied inversely with the concentration of digitalis glycoside in contact with the embryonic hearts. For example, 18 hearts exposed to a concentration of 0.00005 mg of Lanatoside C per cc, beat for an average period of 39 minutes before exhibiting A-V block. On the other hand, hearts exposed to concentrations of 0.002 mg per cc or higher, almost immediately exhibited the acceleration of beating previously noted³ in hearts affected by glycoside and exhibited A-V block much sooner. As a matter of fact, the embryonic hearts, exposed to 0.10 mg of glycoside per cc exhibited A-V block in 15 seconds or less. It is possible that the so-called latent period observed in previous studies may be explained as delay attendant to the penetration of digitalis glycoside into the cells of the adult heart.

Conclusion. The evidence obtained from the actions of Lanatoside C upon the embryonic duck heart indicated that such a heart exhibits a "digitalis effect" with no fixed latent period as described in previous studies. It would appear that if a sufficient quantity of digitalis glycoside is present, a "digitalis effect" may be expected almost immediately.

³ Friedman, M., and Bine, R., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 162.

⁴ Friedman, M., and Bine, R., Jr., *Am. J. Med. Sci.*, 1947, **214**, 633.

Studies of Fluorocardiography in Normal Subjects.

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In previous notes,^{1,2,3} the patterns of the tracings which can be obtained in normal subjects by means of fluorocardiography (electrokymography) have been outlined. The present note reports supplementary observations which deal with the following problems: the tracing of the ascending aorta, the tracing of the pulmonary veins, and the velocity of the pulse wave in the pulmonary circulation. Also the origin and shape of the pulsations of the lung parenchyma are discussed.

Technique. The observations were made by using a Sanborn apparatus for fluorocardiography (electrokymography), and a Sanborn Stethocardiette for phonoelectrocardiography. As previously reported, fluorocardiography records on a continuous film, moving at the speed of 75 mm per second, the pulsations of various areas of the cardiovascular silhouette or the opacity changes of a pulmonary field on x-ray. This is made possible by the use of the x-ray machine, a phototube, a diaphragm with a slit-like opening, a small fluorescent screen, and various stages of amplification. The tracing is recorded by the galvanometer of the Stethocardiette while a simultaneously recorded phonocardiogram permits exact timing of the fluoroscopic pulsations.

The observations were made on 15 normal subjects between the ages of 16 and 30. A total of 10 observations of the pulmonary veins, 10 observations of the ascending aorta, and 15 observations on velocity of the pulmonary arterial wave were performed.

The ascending aorta was studied in the 10 degree and 45 degree left oblique positions.

The slit was placed first over the lowest point above the shadow of the right auricle showing an arterial pulsation, then it was moved step by step toward the arch.

The pulmonary veins were studied by applying the slit transversely over an area about 2 cm beyond the convexity of the right auricle in the postero-anterior position. The lung markings are essentially due to the shadows of the pulmonary vessels. The patterns of the arterial and venous ramifications are generally identical, except for the hilar regions; therefore, arterial and venous shadows cannot be identified as such on the routine roentgenograms. The stronger arterial pulsations overshadow the weaker venous pulsations; therefore the hilar pulsations, visible with the naked eye in certain instances (hilar dance), and the pulsations of the pulmonary parenchyma recorded by fluorocardiography, are mainly arterial. However, the arrangement of the larger vessels in the hilar regions is different for the arteries and veins. It has been known that veins coming from the middle and lower lobes on the right, in their almost horizontal course, cross the downward running arteries perpendicularly and stand out within the bright band of the lower trunk bronchus; they are visible sometimes on routine chest roentgenograms (Assman⁴) and more often on laminograms taken at the appropriate depth (Chatton and Maleki⁵).

Marchal⁶ identified these as vascular shadows of pulmonary veins by injecting an opaque medium intravenously and observing a definite time difference of its arrival at the hilar arteries and at these vessels. These

¹ Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 336.

² Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 348.

³ Luisada, A. A., and Fleischner, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1948, **66**, 436.

⁴ Assman, H., *Die Klinische Röntgendiagnostik der Inneren Erkrankungen*, 5th Ed., Vogel, Berlin, 1934.

⁵ Chatton, P., and Maleki, A., *J. de Radiol. et d'Electr.*, 1947, **28**, 285.

⁶ Marchal, M., *Arch. Mal. Coeur*, 1946, **39**, 345.

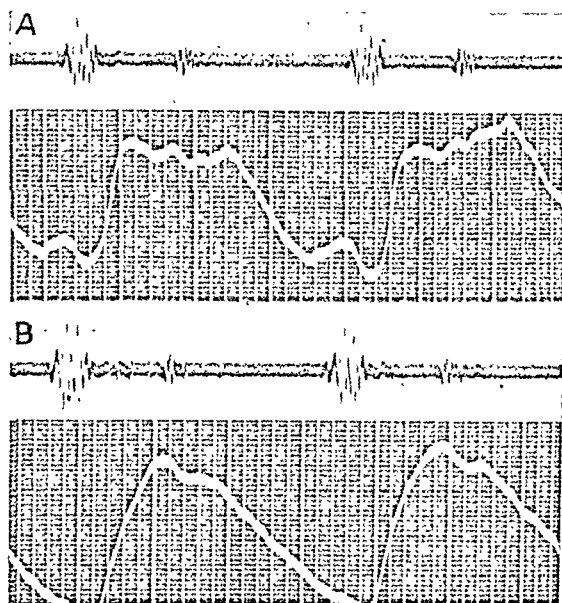


FIG. 1.
Aortic tracings in a normal adult in a 10° left anterior oblique position. (A) Ascending aorta. (B) Aortic arch.

faint vascular shadows can be identified only occasionally by fluoroscopy. However, information gained from Herrnheiser's detailed studies⁷ on the hilar shadows simplified the procedure. In addition to these horizontally crossing veins (*Vena costalis* of the middle lobe and *Vena apicohorizontalis* of the lower lobe), the *Venae basalis posterior* and *axillo-basalis posterior* cross the bright cardio-hilar interspace obliquely, while only one artery of sizable caliber is found in this area. This being an area with predominantly venous vascular shadows, tracings were obtained by us with unexpected frequency, which presented a distinct venous characteristic, namely a presystolic positive wave.

To identify this venous pattern and to rule out incidental waves due to positional movements into and out of the field of observation of arteries or other structures, the following test was performed. The slit was placed vertically upon the border of the right auricle. In successive steps of a few millimeters each, the slit was moved laterally across the bright cardio-hilar interspace, the final tracing being

taken with the slit right upon the arterial hilar trunk. In addition, routine tracings of the right auricle in the postero-anterior view and the left auricle in right oblique view were taken. Thus it became evident that the positive presystolic wave recorded in the "venous field" corresponded temporally to the presystolic left auricular contraction; moreover, there was no evidence of any other pulsatory or positional deflections which might have caused this wave.

The velocity of the pulse waves in the lesser circulation was studied in the following way. Comparison was made of the tracings obtained by placing the slit first over the pulmonary knob, then over the right hilar shadow, and later at the base of the right lung, or better at the lowest visible point above the right diaphragm, following the previously indicated technique.¹ Measurements made in 50 chest films of adults of various sizes and habitus gave the following average data: (a) Distance between pulmonary knob and right hilar shadow, 8 cm. (b) Distance between right hilar shadow and visible base of right lung, 11 cm.

⁷ Herrnheiser, G., *Am. J. Roentg.*, 1942, **48**, 595.

Results and Comments. (1) *The tracing of the ascending aorta* presents a typical pattern which is different from that of tracings recorded over the aortic arch. Its features are as follows (Fig. 1): early systolic drop; rapid rise; early peak; only slight descent (or no descent) during the second half of systole; small incisura; high and occasionally prolonged wave after the incisura; this wave may be higher than the main wave in some cases. This typical tracing resembles certain tracings of pressure recorded by Tigerstedt⁸ on the carotid artery of rabbits while it differs from those recorded by the same author and by Wiggers⁹ in dogs and also from the reconstruction of aortic pressures by Hamilton and Dow.¹⁰ Intra-aortic pressure, recorded by Marey¹¹ in the horse, again presents a more conventional profile. This indicates that the pattern recorded by us in normal human subjects is not merely caused by volume changes (parallel to pressure changes) but is also markedly affected by motions of the heart and vessels. The lowering of the aortic root by ventricular systole and the medial displacement of the ascending aorta by rotation of the heart in the same phase apparently reduce the height of the aortic wave. This is confirmed by the initial drop of the tracing. Opposite movements, taking place in diastole, add their effect to that of the diastolic wave and create a high wave on the tracing. The proximity of the ascending aorta to the left ventricle may contribute to the fact that in some subjects the profile of the aortic pulse during systole greatly resembles a tracing of intraventricular pressure.

(2) *Tracing of the Pulmonary Veins.* Tracings recorded in the right intercardio-hilar space as suggested by Marchal⁶ and discussed above show close relation to those of the auricles. They reveal a presystolic positive

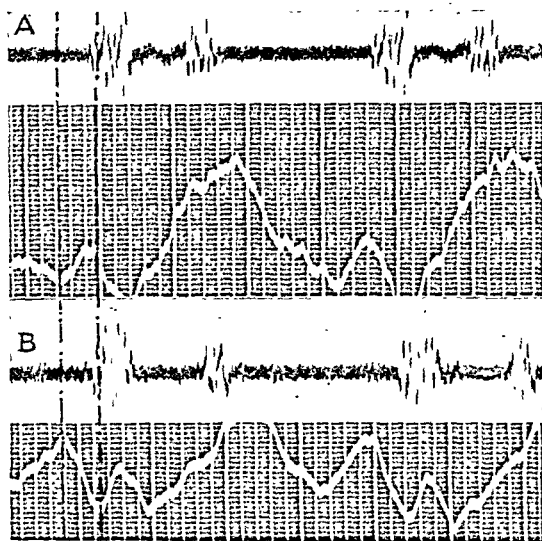


FIG. 2.

Tracing of the pulmonary veins in postero-anterior position (A) compared with that of the left auricle in left oblique (B). Normal subject. The presystolic drop of the auricular tracing is matched by a presystolic rise in the venous tracing.

wave (increased volume) synchronous with the negative wave (contraction or decreased volume) of the left auricular tracing. Such unequivocal records were not obtained in every single case, apparently because of anatomical variability and resulting technical difficulty in placing the slit at the right spot.

(3) *Velocity of the pulse wave in the pulmonary circulation.* While examples of the tracings are given in Fig. 3, the average data obtained in 10 subjects are summarized in the following table. This also gives the maximum and minimum variations and the range of physiological variations on the basis of 8 cases out of 10, disregarding exceptional variations.

For greater accuracy, the distances between 1st sound and foot of the pulse waves were measured by using the beginning of the 1st sound vibrations but not that vibration which marks the opening of the semilunar valves.¹² This gives a prolongation of the absolute individual temporal relationship of about 0.05 seconds. However, it fails to change the

⁸ Tigerstedt, R., *Die Physiologie des Kreislaufes*, 2nd Ed., DeGruyter, Berlin, 1922, 3, p. 213.

⁹ Wiggers, C. J., *Modern Aspects of the Circulation in Health and Disease*, Lea and Febiger, Phila., 1923.

¹⁰ Hamilton, W. F., and Dow, P., *Am. J. Physiol.*, 1939, 125, 48.

¹¹ Marey, E. J., *La Méthode Graphique dans les Sciences Expérimentales*, Masson, Paris, 1885.

¹² Rappaport, M. B., and Sprague, H. B., *Am. Heart J.*, 1942, 23, 591.

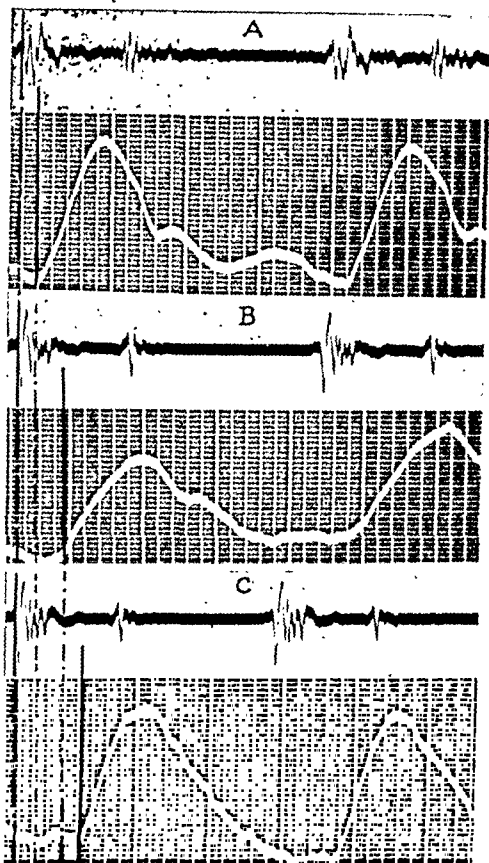


FIG. 3.

Tracing of the pulmonary knob (A), of the right hilar shadow (B), and of the visible base of the right lung (C) in a normal subject. Postero-anterior position.

relative intervals because all figures are obtained in the same way. As indicated in the table, the average times of arrival of the pulse waves are 0.08 sec. for the pulmonary knob, 0.12 sec. for the right hilus, and 0.16 sec. for the visible base of the right lung. Using the average figures of 8 and 11 cm indicated above, we obtain a velocity of the pulse waves which is 2 meters per second between pulmonary knob and right hilus and 2.75 meters per second between right hilus and visible base of the right lung. These speeds should be compared with the known speeds of the pulse waves in the greater circulation. These vary between 5 and 9 meters per second in normal subjects and are greater in the peripheral vessels (7.5) than in the aorta

(4.5).^{13,14}

Therefore, while the speed of the pulse in the lesser circulation is lower than in the greater, being roughly one third of the latter, the pulse increases its speed in the small, less extensible arterioles both in the lesser and in the greater circulation. The lower velocity of the pulse wave in the pulmonary circulation is caused by the lower pressure and by the greater extensibility of the vessels. This fact had been demonstrated in experimental animals and appears now confirmed for man.

(4) *Shape and cause of the pulmonary wave.* The peak and the shape of the pulsatory wave of the lung have been the object of special attention. The peak has a less fixed position in the tracing of the pulmonary parenchyma than in that of the hilus. In spite of that, it is easy to see that the peak in the pulmonary tracing is nearer to the foot than in the hilar tracing; it is followed by a more rapid descent; it often follows closely that small vibration of the 2nd sound which marks the opening of the mitral valve.¹⁵ For these reasons, we came to the conclusion that the tracing of the pulmonary parenchyma is similar to a plethysmogram and is modified by both arterial and venous changes in the blood content of the lung. The rise and the ascending part of the wave are of arterial origin, as shown by the respective time relationships that they have with similar waves of the hilar tracing and of the tracing of the pulmonary knob. On the contrary, the descending branch of the wave reflects mainly changes in venous tracing. It is possible that occasionally some venous component is present in the hilar tracing too but this is less likely for anatomical reasons and because usually much less amplification is required for recording that tracing. Therefore, possible venous components are minimized by the technique used.

¹³ Bazett, H. C., and Dreyer, N. B., *Am. J. Physiol.*, 1922, **63**, 94.

¹⁴ Wiggers, C. J., *Ann. Int. Med.*, 1932, **6**, 12; *Am. Heart J.*, 1938, **16**, 515.

¹⁵ Hamilton, W. F., *Circulation Through Special Regions*, in *Howell's Textbook of Physiology* edited by J. F. Fulton, W. B. Saunders, Phila., 1946.

TABLE I.
Velocity and Duration of Pulmonic Arterial Waves.

	Avg distance from 1st sound to foot of pulse wave	Distance between foot and peak of the same wave	Transmission time	Speed of the wave
Pulmonary knob	0.08 sec. ± 25% Mx = 0.10 Mn = 0.05	0.14 sec.		
Right hilar shadow	0.12 sec. ± 25% Mx = 0.15 Mn = 0.09	0.24 "	Pulm. knob to rt. hilus 0.04 sec.	2 M/sec.
Right lung (visible base)	0.16 sec. ± 25% Mx = 0.22 Mn = 0.10	0.23 "	Rt. hilus to visible base of rt. lung 0.04 sec.	2.75 M/sec.

Summary. A study of 15 normal subjects has been performed by using fluorocardiography, a method which permits recording on a continuous film of the pulsations of various cardiovascular structures revealed by the x-ray. (a) The tracing of the ascending aorta presents a typical pattern which partly is due to transmission of intraventricular pressure and partly to motions of the aortic root connected with ventricular systole and diastole. (b) It is possible to record in certain cases a tracing of the pulmonary veins. Its most typical feature is a positive wave

during presystole. (c) The velocity of the pulse wave in the lesser circulation is much lower than in the greater circulation. It is more rapid in the smaller branches than in the stems of the pulmonary artery. Average figures are given. (d) The tracing of the pulmonary parenchyma is the equivalent of a plethysmogram. While the rise and the ascending branch of the wave are of arterial pulmonary origin, the peak and the descending branch of the wave are probably of venous pulmonary origin.

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*Bacillomycin: An Antibiotic from Bacillus subtilis Active against Pathogenic Fungi.**

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Since the discovery of *subtilin* by Jansen and Hirschmann,¹ 3 additional antibiotics i.e., *bacitracin*, *bacillin*, and *eumycin* have

* The authors wish to express their indebtedness to Dr. E. G. Snyder and R. W. Whitley for purification and chemical studies; and to Miss Charlotte Campbell, Army Medical Department Research and Graduate School, for the systemic fungi spectrum.

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been described as products of strains of *Bacillus subtilis* (for an excellent, comprehensive review see Benedict and Langlykke²). With the exception of *eumycin*, the antibiotics possess incidental antifungal properties in association with a more specific antibacterial action.

¹ Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, 4, 297.

² Benedict, R. G., and Langlykke, A. F., *Ann. Rev. Microbiol.*, 1947, 1, 193.

TABLE I.
Antifungal Spectrum of Bacillomycin.

Organism	Source of strain	mg of Bacillomycin per ml of medium									
		0.5	0.25	0.10	0.05	0.025	0.010	0.005	0.0025	0.001	Control
Dermatophytes:											
<i>Epidermophyton floccosum</i>	Univ. of Penn.	—	—	—	—	—	+	+	+	+	+
<i>Microsporum audouinii</i>	" " "	—	—	—	—	—	+	+	+	+	+
<i>Microsporum gypsum</i>	" " "	—	—	—	—	—	+	+	+	+	+
<i>Trichophyton mentagrophytes</i>	A.T.C.C.	—	—	—	—	—	+	+	+	+	+
<i>Trichophyton rubrum</i>	" "	—	—	—	—	—	+	+	+	+	+
<i>Trichophyton schoenleinii</i>	Univ. of Penn.	—	—	—	—	—	+	+	+	+	+
Systemic Fungi:											
<i>Blastomyces dermatitidis</i> (Mycelial)	Duke Univ.	—	—	—	—	—	—	R	+	+	+
<i>Blastomyces dermatitidis</i> * (Yeast form)	" "	—	—	—	—	—	—	—	+	+	+
<i>Candida albicans</i>	Army Med. Sch.	—	—	—	—	R	+	+	+	+	+
<i>Coccidioides immitis</i>	" "	—	—	—	—	—	+	+	+	+	+
<i>Torula histolytica</i>	Naval Med. Center	—	—	—	—	R	+	+	+	+	+
<i>Histoplasma capsulatum</i>	Duke Univ.	—	—	—	—	—	+	+	+	+	+
<i>Hormodendrum pedrosoi</i>	Univ. Brazil	—	—	—	—	—	+	+	+	+	+
<i>Monosporium apiospermum</i>	Duke Univ.	R	R	+	+	+	+	+	+	+	+
<i>Nocardia asteroides</i>	Army Med. Sch.	+	+	+	+	+	+	+	+	+	+
<i>Phialophora verrucosa</i>	Nat. Inst. Health	R	+	+	+	+	+	+	+	+	+
<i>Sporotrichum schenckii</i>	Duke Univ.	—	—	—	—	R	+	+	+	+	+
<i>Blastomyces brasiliensis</i>	" "	—	—	—	—	—	+	+	+	+	+

Cultures were incubated at 30°C for 15-17 days. Medium was Sabouraud maltose agar.
* Inoculated from a culture in "yeast" phase and incubated at 37°C for 20 days.
Key: + = growth, R = growth restricted; — = no growth.

In the course of a program designed to find antibiotics active against *Torula histolytica* infections, there was isolated from a contaminated *Actinomyces griseus* culture a strain of *Bacillus subtilis* which possessed negligible antibacterial activity and striking fungistatic properties. Although concentrates of this antibiotic and *eumycin*^{3,4} showed a spectrum similar in many respects, the limited available data on the chemistry of *eumycin* as well as its specific action on *Corynebacterium diphtheriae* and acidfast bacilli appeared to differentiate it from our antibiotic and warranted further study. The antibiotic substance has been designated *bacillomycin*.

Bacillomycin occurs in the cell-free fermentation liquor after cultivation of the organism for 2 to 3 days in shake culture or 5 to 6 days in surface culture at 24-28°C in a synthetic medium composed of 20 g glucose, 5 g l-glutamic acid, 0.5 g MgSO_4 , 0.5 g KCl, 1 g KH_2PO_4 , 0.15 mg $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$, 5.0 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.16 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1000 ml distilled water; final pH is 6.0. Maximal yields of the product are obtained with a rise in pH of the medium to 7.0-7.6.

Crude filtrates are assayed against a standardized spore suspension of *Trichophyton mentagrophytes* by the agar cup method on Sabouraud's maltose agar. Standard solutions and experimental samples are tested in duplicate on each plate and the latter incubated at 30°C for 72 hours. The unit of activity is equivalent to the zone produced by 0.1 mg of a standard preparation of *bacillomycin*. This represents a zone of 20 to 25 mm.

The antifungal activity of partially purified *bacillomycin* was measured by the agar plate-dilution method. The results are reported in terms of the smallest amount of the antibiotic in a constant volume of test medium (10 ml) which will give complete inhibition of the test organism. Incubation of the tests

(with one exception) was at 30°C for 15-17 days. A summary of the inhibition experiments on a spectrum of pathogenic fungi is compiled in Table I.

Bacillomycin is precipitated from the broth by acidifying to pH 2.5 with hydrochloric acid solution. The precipitate is extracted with ethanol, washed with ether and dried *in vacuo* over phosphorus pentoxide. (The ether step assumes importance in the extraction since an additional antibiotic elaborated by the organism and active against gram positive bacteria is also precipitated at pH 2.5. However, whereas the antifungal product is largely ether insoluble, the antibacterial activity is soluble.) The activity averages 5 units/mg and the yield is approximately 0.3 g/liter broth with a 35 to 50 % recovery.

Bacillomycin is soluble in methanol, ethanol, n-butanol and acetone. It is precipitated by concentrated neutral ammonium sulfate solution. The active substance is readily adsorbed on activated charcoal but is difficult to elute. The antibiotic will not dialyze through cellophane membranes.

Bacillomycin is not destroyed by trypsin or pepsin. The product is stable in dried form. Samples stored at room temperature have retained their activity for months. Solutions adjusted in pH to include acid (3.0) neutral and alkaline (9.0), may be autoclaved (120°C-20 minutes) with no appreciable loss of activity.

Summary. A strain of *Bacillus subtilis* has been isolated which elaborates a previously undescribed antibiotic "Bacillomycin." This antibiotic possesses striking antifungal activity and almost complete lack of antibacterial action. The fungus spectrum of "Bacillomycin" includes practically all of the important dermatophytes and systemic fungi. A satisfactory bioassay employing the agar cup plate technic and a spore suspension of *Trichophyton mentagrophytes* is described. Some physical and chemical properties of the antibiotic are given together with a simple procedure for its concentration from culture broths.

³ Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

⁴ Burdon, K. L., and Johnson, E. A., Conference on Antibiotic Research, Antibiotics Study Sect., Nat. Inst. Health, 1947.

Renal Tubular Secretion of Potassium in the Normal Dog.*

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During the administration of salyrgan[†] (sodium salt of mercury salicyl-allylamide-ortho-acetate) to dogs, it was observed that the rate of potassium excretion frequently became constant and remained at a fixed level despite marked changes in the calculated rate of potassium filtration at the glomerulus. A constant excretory rate dissociated from filtered load is strongly suggestive of a tubular secretory mechanism. Such a mechanism for the addition of potassium to the tubular urine has, in fact, been demonstrated in the dog by experiments to be described.

Material and Methods. Experiments were performed on 4 trained, unanesthetized female dogs. To obtain stable plasma creatinine and inulin concentrations and to assure constant rates of potassium intake, solutions were administered by continuous infusion. Urine and heparinized venous blood samples[‡] were collected by the usual techniques for the determination of clearances.

The plasma creatinine clearance was used as a measure of glomerular filtration rate. The equivalence of the creatinine clearance and filtration rate in the dog is generally accepted. In 2 experiments inulin clearances were simultaneously determined to check on the validity of the creatinine clearance as a

measure of filtration rate under the circumstances of these experiments.

Creatinine was determined in tungstic acid filtrates of plasma and in diluted urine by a modification of the Folin method.¹ Inulin was determined by a modification of Harrison's method.² Both plasma and urine were treated with yeast before precipitation with zinc.

Potassium and sodium in plasma and urine were determined with an internal standard flame photometer,³ after addition of a standard amount of lithium to each specimen and dilution. The error of the method in our hands does not exceed 1% in the recovery of added amounts. The presence of protein in diluted plasma samples did not interfere with the determination since ashed specimens gave results identical with those obtained by simple dilution. The addition of amounts of sodium greater than those present in plasma samples was found not to affect potassium determinations.

Results. An experiment showing the effect of salyrgan on potassium excretion is summarized in Table I. Soon after the administration of salyrgan the rate of excretion of potassium reached a value of about 45 μ eq/min and remained at this level for a period of more than 2 hours despite a fall of 35% in the filtered potassium and during marked changes in urine flow and sodium excretion. Similar results were obtained in each of 3 other dogs. A slight rise in potassium excretion with time was sometimes observed in

* The work described in this paper was supported in part by grants provided by the National Institute of Health (U.S.P.H.S.) and from the Josiah Macy, Jr., Foundation.

† The salyrgan used in this study was supplied by the medical research department of Winthrop-Stearns, Inc.

‡ Arterial blood samples were simultaneously obtained in several instances. The concentration of potassium in arterial plasma did not differ measurably from that in venous plasma during the infusion of KCl.

¹ Shannon, J. A., and Fisher, S., *Am. J. Physiol.*, 1938, 122, 765.

² Harrison, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 111.

³ Berry, J. W., Chappell, D. G., and Barnes, R. B., *Ind. Eng. Chem., Anal. Ed.*, 1945, 17, 605.

TABLE I.
Effect of Salyrgan on Potassium Excretion. Dog F. 13.9 Kilos.

Clearance period	Time, min.	Plasma concentration*			Urine flow, ml/min	Creatinine clearance, ml/min	"Filtered"†		Excreted	
		Creatinine, mg %	Sodium, meq/L	Potassium, meq/L			Sodium, μ eq/min	Potassium, μ eq/min	Sodium, μ eq/min	Potassium, μ eq/min
	0		148	3.7						
	Priming creatinine 0.9 g in 200 ml isotonic NaCl.									
	Start infusion 0.45% creatinine in isotonic NaCl at 2 ml/min.									
1	18-38	10.8	149	3.6	0.62	59	8800	212	105	29
2	38-59	11.6	149	3.5	0.72	58	8600	203	93	19
	62		Salyrgan 1 ml I.V. 3.2 ml Salyrgan added to 400 ml of infusion.							
3	81-104	12.6			8.89	67	10000	228	1410	31
4	104-115	12.5	149	3.4	10.03	81	12100	275	1860	44
5	115-131	12.3			6.00	60	10300	234	1270	48
6	180-196	13.8			2.12	53	7800	175	350	45
7	196-211	14.5	148	3.3	2.23	52	7700	172	353	45
8	211-232	—			2.90	—	—	—	440	46

* Plasma samples obtained at midpoint of corresponding clearance periods.

† Plasma concentration times creatinine clearance; uncorrected for Donnan equilibrium.

these experiments. The rate of potassium excretion after salyrgan was nearly constant in each experiment but varied from dog to dog, 45 μ eq/min being the lowest rate observed while 150 μ eq/min was the highest. Salyrgan did not always effect an increase in potassium excretion. When potassium excretion was initially increased by administration of KCl, salyrgan produced a decrease in the excretion rate.

The most direct evidence for a secretory mechanism for potassium would be the demonstration of more potassium in the urine than could be accounted for by glomerular filtration. Since the amount secreted would probably be small in relation to the amount which might be filtered, it was to be expected that some difficulty would be encountered in demonstrating a secretory mechanism. However, in each of the 4 dogs it has been possible to obtain at least one experiment in which the excreted potassium exceeded that filtered. Only in the dog with the largest potassium excretion after salyrgan was this achieved in the first attempt. In the other dogs several preliminary experiments were sometimes necessary to determine the optimum infusion rate for demonstration of this phenomenon. Achievement of the necessary conditions seemed to be facilitated by the preliminary oral administration of KCl⁴ for a week before the experiment and by the administration of the KCl during experiments in hypertonic solution and at a moderate rate. One such experiment is shown in Table II. In this experiment, within 40 minutes of the time that the infusion rate was increased to 0.67 meq/min, the excreted potassium exceeded that "filtered" by 25% and ratios of excreted to "filtered" varied between 1.15 and 1.33 in 9 successive clearance periods. Inulin clearances were determined in 2 similar experiments, one of which is shown in Table III. In both experiments the inulin clearances corresponded closely with simultaneously determined creatinine clearances. In the 2 other dogs, ratios of excreted to "filtered" potassium greater than one were obtained

⁴ Thatcher, J. S., and Radike, A. W., *Am. J. Physiol.*, 1947, 151, 138.

in 22 clearance periods in 3 experiments; the ratios reached at least 1.15 in each dog. It is worthy of note that after preparation with oral KCl high rates of potassium excretion were attained with only minimal elevation of the plasma potassium concentration (Table III).

Discussion. The excess of excreted potassium over filtered load was well beyond the limits of experimental error. There is no reason to believe that the filtration rate actually exceeded the creatinine clearance, especially since the inulin and creatinine clearances were the same.

It should be noted that the amount of potassium filtered has been calculated simply as the product of creatinine clearance and plasma potassium. Correction of the filtered potassium for the Donnan equilibrium would lower the filtered load by about 5% and increase the excess of excreted potassium observed in these experiments.

The data obtained constitute evidence for the existence of a mechanism for the addition

of potassium to the tubular urine.[§] The coexistence of tubular mechanisms for both reabsorption and secretion of a single substance has not previously been demonstrated. Tubular secretion of a number of substances generally considered to undergo only filtration and reabsorption, including potassium, has been suggested by Barclay, Cooke and Kenney,⁶ but the evidence on which this conclusion is based is not presented in the published abstract.

Summary and Conclusions. A constant rate of potassium excretion, dissociated from filtered load, occurring after salyrgan administration suggested a tubular secretory mechanism located, presumably, in the distal tubule. The presence of such a mechanism has been demonstrated by the intravenous administration of hypertonic KCl solutions which yielded rates of potassium excretion considerably above the rates of filtration of potassium at the glomerulus.

[§] Mudge, G. H., Foulks, J. G., and Gilman, A., personal communication.

⁶ Barclay, J. A., Cooke, W. T., and Kenney, R. A., XVII Internat. Physiol. Congress, Abstracts of Communications, 1947, p. 58.

[§] Doctors Mudge, Foulks, and Gilman[§] inform us that they have similarly concluded that potassium is secreted by the renal tubules on the basis of observations during forced osmotic diuresis.

16369 P

The Renal Excretion of Potassium.*

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The potassium (K) excreted in the urine under normal circumstances can be accounted for by the tubular rejection of approximately 10% of the calculated filtered K. This has been accepted as evidence that K is excreted by the process of filtration and incomplete reabsorption. The possibility of tubular se-

cretion has been suggested by isolated observations. McCance and Widdowson¹ reported a case of alkalosis and dehydration with a low filtration rate and a K clearance greater than the inulin clearance. Keith, King and Osterberg² noted similar K : Inulin clearance

* This study has been supported by a grant from the United States Public Health Service.

[†] National Research Council Fellow.

¹ McCance, R. A., and Widdowson, E. M., *Lancet*, 1937, 2, 247.

² Keith, N. M., King, H. E., and Osterberg, A. E., *Arch. Int. Med.*, 1943, 71, 675.

ratios in normal subjects receiving KCl, but gave no experimental data and did not mention possible K secretion in subsequent reports.³ Barclay, Cooke and Kenney⁴ stated that K, urea and phosphorus were excreted by the triple process of filtration, reabsorption and secretion, but gave no experimental results. Winkler and Smith⁵ observed a maximum excretion of 35% of filtered K in dogs, but noted no evidence of tubular secretion. This preliminary report is submitted because in experiments on dogs we have repeatedly observed K clearances greater than the simultaneously determined filtration rates. In studies on trained dogs infused with KCl, Berliner and Kennedy⁶ have also independently observed K excretion in excess of the calculated amount filtered.

Methods. Mongrel dogs were studied under light pentobarbital anesthesia. Conventional clearance techniques and analytical procedures were employed. Creatinine or thiosulfate clearances were used as a measure of the glomerular filtration rate. K was determined by an improved type internal standard flame photometer with an accuracy of $\pm 1\%$. K clearances were calculated without correction for the Donnan equilibrium or protein binding.

Results. In studies on the electrolyte excretion associated with urea diuresis, K clearances increased from 5-10% of the creatinine clearance at normal rates of urine flow to 80-90% of the creatinine clearance during marked diuresis with minute urine volumes of 18 to 35 cc and at creatinine U/P ratios of approximately 2. At lower creatinine U/P ratios the Ck/Ccr ratio exceeded 1 by as much as 0.36 without the administration of exogenous K. Under these circumstances the urea clearance/creatinine

TABLE I.
Effect of Urea Diuresis on the U/P Ratios of Creatinine and Potassium.

Elapsed time, min.	KCl infusion rate, mEq/min.	Urine vol., cc/min.	Serum K, mEq/L	Serum urea, mOsm/L	C _{urea} / C _{cr}	U — P	U — K P	U/P K U/P Cr
10	0	1.7	4.4	2.9	.58	27.9	2.4	.09
20	KCl infusion started.							
160	.450	8.2	10.3	2.3	.72	7.6	4.6	.60
161	50% urea infusion started at 12.5 mOsm/min.							
180	.450	9.2	10.4	15.8	.66	5.02	3.7	.74
235	.350	—	10.6	—		1.41	1.53	1.09
255	.163	22.5	7.3	276	.88	1.82	2.37	1.30
275	.300	12.2	6.9	247	.81	2.15	2.99	1.39
295	.300	14.4	8.9	326	.85	1.33	1.60	1.20
310	.500	—	10.6	—	—	1.34	1.50	1.12

³ Keith, N. M., and Osterberg, A. E., *J. Clin. Invest.*, 1947, **26**, 773.

⁴ Barclay, J. A., Cooke, W. T., and Kenney, R. A., *XVII Internat. Physiol. Congress, 1947, Abstracts*, page 58.

⁵ Winkler, A. W., and Smith, P. K., *Am. J. Physiol.*, 1942, **138**, 94.

⁶ Berliner, R. W., and Kennedy, J. P., personal communication.

TABLE II
Effect of NaHCO_3 on the Renal Clearance of Potassium and Thiosulfate.

Elapsed time, min.	Urine vol., cc/min.	Serum K, mEq/L	Urine pH	Clearance	Clearance K cc/min.	C_K
				S_2O_3 cc/min.		$C_{S_2O_3}$
—143	Start of constant infusion .375 mEq KCl/min., maintained during entire experiment.					
0-20	3.8	7.3	5.6	56.8	67.5	1.19
40-60	1.8	6.9	5.6	55.7	53.4	.96
80-100	2.0	5.8	5.4	52.7	49.4	.94
120-140	2.6	6.3	5.6	51.2	46.8	.92
150-170	3.1	6.7	5.6	53.1	47.7	.90
	I.v. infusion of 200 mEq $NaHCO_3$ in 1 L. in 35 min.					
210-230	15.7	5.0	7.8	54.0	65.8	1.22
235-255	8.5	4.7	7.8	52.6	67.3	1.28
260-280	6.6	4.7	8.1	55.5	60.3	1.09

clearance ratios averaged .83 and never exceeded 1.0. In the sample protocol (Table I) both K and urea were infused and due to ECG changes the K infusion rate was not constant and therefore "spot" U/P ratios were taken.

Because of the elevation of urine pH occurring during marked urea diuresis and because of the observation of Darrow⁷ that alkalosis is associated with K depletion when renal function is normal, the effect of alkalosis was studied in dogs receiving KCl by intravenous infusion. Under these conditions K clearances greater than filtration rates were also demonstrated (Table II).

C_K/C_{cr} ratios above 1 were observed in 7 different dogs with filtration rates varying from 23 to 75 cc per minute. K excretion in excess of filtration was not observed in every experiment. Factors which favored C_K/C_{cr} ratios greater than 1 were: (1) a moderate, rather than marked elevation of serum K when KCl was infused, (2) a decline in filtration rate which usually accompanied extreme urea diuresis, and (3) the duration of the

KCl infusion. The first two factors would help reveal a secretory mechanism in the face of partial reabsorption of K. The third may represent a physiological adjustment.

Discussion and Conclusions. K clearances in excess of creatinine or thiosulfate clearances, under the conditions of these experiments, are interpreted as evidence that all of the K excreted in the urine cannot be accounted for by the filtration-reabsorption theory alone, but that tubular secretion of K also occurs. In the experiments on urea diuresis, urea clearances never exceeded creatinine clearances, and the ratios agreed with the observations of Shannon.⁸ It is a reasonable hypothesis that during urea diuresis the secretion of K becomes apparent because its normal tubular reabsorption is hindered. The present data are insufficient to define the mechanisms involved in the secretory process or to demonstrate the relative magnitude of the processes of filtration, reabsorption and secretion in the renal excretion of K. Further work is in progress.

⁸ Shannon, J. A., *Am. J. Physiol.*, 1938, **122**, 782.

⁷ Darrow, D. C., *J. Clin. Invest.*, 1946, **25**, 324.

Differences in Sera of Human Subjects with Respect to Heteroagglutinins for Mouse Erythrocytes.*

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In attempting to demonstrate and induce agglutinins in the blood of 9 types of hybrid mice and in mice of 5 strains inbred for many generations, suspensions of mouse erythrocytes in human sera were routinely used as control material.¹ It was thus noticed that mouse erythrocytes were agglutinated by the sera obtained from human subjects of all 4 blood groups. There seemed to be no special affinity of the erythrocytes of mice for sera of any one type as such. It was apparent, however, that not all the samples of sera from human subjects possessed the property of agglutinating mouse erythrocytes. The negative reactions were heterospecific with respect to the blood groups in human subjects. The most obvious conclusion, therefore, was that there existed an antigen in mouse erythrocytes, the serological analog of which was present in most, but not all, human sera. Subsequent experiments showed that this serological component was specifically adsorbed by homologous antigen. For the sake of convenience, "Mo agglutinin" will henceforth be used to designate this component of human serum.

Gorer²⁻⁵ demonstrated that isoagglutinins were present in the sera of mice in which tumors had recently regressed. This work was independently duplicated by Lumsden⁶ who induced isoagglutinins in rats by means of normal or malignant nucleated cells. Both Gorer³ and Lumsden⁶ concluded that the natural resistance of a rat or a mouse to a tumor transplantation is directly proportional

to the capacity of the animal to produce agglutinins.

In this paper we have been concerned with an agglutinin for mouse erythrocytes which occurs in most human sera and which is similar to or identical with that developing in mouse sera when transplanted tumors regress. While this is probably not related to the factors that govern susceptibility to cancer in the human subject, it is of interest that it was not present in approximately 14% of human subjects. It is the purpose of the present paper to present the work on the attempts to determine the approximate incidence and data on the adsorption and specificity of the Mo agglutinin in human sera.

Method and Materials. In these experiments, mice[†] of the following inbred strains were employed: C₃H, C₅₇ black, I, A, and JK. The following types of hybrid mice of F₁ and F₂ generations were also used: C₅₇x C₃H, C₃Hx C₅₇, C₃HxA, C₅₇x CBAN, C₅₇CBANxA, C₅₇xA, AxCBAN, Ax C₅₇CBAN. In general, the technique used was similar to those described by Gorer.² The samples of human sera were obtained commercially, or from ourselves, our colleagues, and the Baltimore Rh Typing Laboratory. All sera were inactivated by heating in a water bath at 56°C for 30 minutes. 1:5000 merthiolate solution was used as a preservative.

The agglutination tests were carried out as follows: One drop of blood was taken from the mouse tail and suspended in 2 cc saline-citrate solution. The sera were diluted 1:2. Two drops of diluted serum and 4 drops of cell suspension were placed in a small test tube and mixed. The tubes were then centrifuged slowly for 30 seconds and then

* This work was aided by grants from the Anna Fuller Fund and the Donner Foundation.

¹ Figge, F. H. J., Cohen, L., and Winokur, G., submitted for publication.

² Gorer, P. A., *J. Gen.*, 1936, **32**, 17.

³ Gorer, P. A., *Brit. J. Exp. Path.*, 1936, **17**, 42.

⁴ Gorer, P. A., *Brit. J. Exp. Path.*, 1937, **18**, 31.

⁵ Gorer, P. A., *J. Path. and Bact.*, 1937, **44**, 691.

⁶ Lumsden, T., *Am. J. Cancer*, 1938, **32**, 395.

[†] All the mice used in these experiments were the progeny of mice obtained from Dr. L. C. Strong in 1941.

TABLE I.
Agglutination of Mouse AxCBAN (7) Erythrocytes with Various Human Sera.
(Numbers in parentheses are for identification of sera.)

A (1)	3+	A (57)	3+	B (21)	2+	AB (20)	3+	O (65)	2+
A (46)	2-3+	A (58)	2+	B (22)	—	AB (277)	3+		
A (50)	2+	A (59)	2+	B (23)	3+	AB (141)	3+		
A (51)	2-3+	A (60)	1+	B (24)	3+	AB (142)	—		
A (52)	3+	A (61)	2+	B (25)	2+	O (60)	2+		
A (53)	2+	A (62)	2+	B (26)	3+	O (61)	3+		
A (54)	—	A (63)	3+	B (27)	3+	O (62)	3+		
A (55)	2+	A (64)	3+	AB (1)	3+	O (63)	—		
A (56)	3+	B (1)	2+	AB (10)	3+	O (64)	—		

allowed to stand for 30 minutes after which they were read with the naked eye and checked microscopically. The readings were recorded as —, 1+, 2+, and 3+, depending on the degree of reaction.

The following adsorption technique was employed: 5 cc of oxalated blood of the desired type was centrifuged and the cells were washed 3 times with physiological saline solution. After the final washing, the supernatant saline was drawn off with a pipette and 10-12 drops of the corresponding serum to be used was added to the cells. The cells and serum were then mixed, centrifuged slowly for 30 seconds, and then allowed to stand 3 hours. The tube was then centrifuged at high speed for 5 minutes, after which the serum was removed and tested against a suspension of the blood cells used in the adsorption. If there was no agglutination of these cells, it was assumed that all the agglutinins had been removed from the serum.

Results. Human type A, B, AB, and O sera were found to agglutinate mouse erythrocytes. The agglutino-gen was invariably present in the erythrocytes of mice of all of the 5 strains inbred and the 8 types of hybrid mice mentioned previously.

The data in Table I show that the Mo agglutinin was not present in 5 of 37 samples (approximately 14%) of human sera. It may also be observed that the negative reactions occurred in each of the four major blood groups so that the absence as well as the presence of the Mo agglutinin is not specifically associated with any of the standard human blood groups.

In the adsorption studies (Table II), the Mo agglutinin was shown to be a specific agglutinin which could be adsorbed from

human serum by mouse erythrocytes, but not by human erythrocytes of any blood group (Table II). It was found to be entirely independent of α and β agglutinins as indicated by the fact that it was present in 4, but absent in 2, sera of the O group (Table I). Moreover, the α and β agglutinins could be adsorbed on the appropriate human erythrocytes without removing the Mo agglutinin. Conversely, the adsorption of the Mo agglutinin on mouse erythrocytes did not remove the α or β agglutinins or a heterospecific agglutinin for rabbit erythrocytes.^{7,8} (Table II.)

The Mo agglutinin was also found to be present in both Rh+ and Rh- blood, and group A Rh+ cells failed to adsorb the Mo agglutinin. This data indicated that there was no apparent relationship between Mo agglutinin and Rh agglutinin. It is, perhaps, needless to point out that Rh sensitization is an induced phenomenon in Rh-persons who have had previous transfusions with Rh+ blood; or in some Rh- mothers who have been delivered of Rh+ children, the total incidence being comparatively low, at least in the latter group; whereas the Mo agglutinin is concerned with a hitherto unnamed naturally occurring agglutinin present in most, but not all, human sera examined thus far.

Discussion. It is necessary to clarify the contribution made by this work in relation to the advances made by others. Sievers⁷ has presented a case of heteroagglutination with human serum and has shown that the agglutinins vary in different species of animals.

⁷ Sievers, O., *Acta Path. et Microbiol. Scand.*, 1937, **14**, 553.

⁸ Wiener, A. S., *Blood Groups and Transfusion*, 3rd ed., C. C. Thomas, Springfield, 1943.

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In attempting to demonstrate and induce agglutinins in the blood of 9 types of hybrid mice and in mice of 5 strains inbred for many generations, suspensions of mouse erythrocytes in human sera were routinely used as control material.¹ It was thus noticed that mouse erythrocytes were agglutinated by the sera obtained from human subjects of all 4 blood groups. There seemed to be no special affinity of the erythrocytes of mice for sera of any one type as such. It was apparent, however, that not all the samples of sera from human subjects possessed the property of agglutinating mouse erythrocytes. The negative reactions were heterospecific with respect to the blood groups in human subjects. The most obvious conclusion, therefore, was that there existed an antigen in mouse erythrocytes, the serological analog of which was present in most, but not all, human sera. Subsequent experiments showed that this serological component was specifically adsorbed by homologous antigen. For the sake of convenience, "Mo agglutinin" will henceforth be used to designate this component of human serum.

Gorer²⁻⁵ demonstrated that isoagglutinins were present in the sera of mice in which tumors had recently regressed. This work was independently duplicated by Lumsden⁶ who induced isoagglutinins in rats by means of normal or malignant nucleated cells. Both Gorer³ and Lumsden⁶ concluded that the natural resistance of a rat or a mouse to a tumor transplantation is directly proportional

to the capacity of the animal to produce agglutinins.

In this paper we have been concerned with an agglutinin for mouse erythrocytes which occurs in most human sera and which is similar to or identical with that developing in mouse sera when transplanted tumors regress. While this is probably not related to the factors that govern susceptibility to cancer in the human subject, it is of interest that it was not present in approximately 14% of human subjects. It is the purpose of the present paper to present the work on the attempts to determine the approximate incidence and data on the adsorption and specificity of the Mo agglutinin in human sera.

Method and Materials. In these experiments, mice[†] of the following inbred strains were employed: C₃H, C₅₇ black, I, A, and JK. The following types of hybrid mice of F₁ and F₂ generations were also used: C₅₇ × C₃H, C₃H × C₅₇, C₃H × A, C₅₇ × CBAN, C₅₇ × CBAN × A, C₅₇ × A, A × CBAN, A × C₅₇ × CBAN. In general, the technique used was similar to those described by Gorer.² The samples of human sera were obtained commercially, or from ourselves, our colleagues, and the Baltimore Rh Typing Laboratory. All sera were inactivated by heating in a water bath at 56°C for 30 minutes. 1:5000 merthiolate solution was used as a preservative.

The agglutination tests were carried out as follows: One drop of blood was taken from the mouse tail and suspended in 2 cc saline-citrate solution. The sera were diluted 1:2. Two drops of diluted serum and 4 drops of cell suspension were placed in a small test tube and mixed. The tubes were then centrifuged slowly for 30 seconds and then

* This work was aided by grants from the Anna Fuller Fund and the Donner Foundation.

¹ Figge, F. H. J., Cohen, L., and Winokur, G., submitted for publication.

² Gorer, P. A., *J. Gen.*, 1936, **32**, 17.

³ Gorer, P. A., *Brit. J. Exp. Path.*, 1936, **17**, 42.

⁴ Gorer, P. A., *Brit. J. Exp. Path.*, 1937, **18**, 31.

⁵ Gorer, P. A., *J. Path. and Bact.*, 1937, **44**, 691.

⁶ Lumsden, T., *Am. J. Cancer*, 1938, **32**, 395.

[†] All the mice used in these experiments were the progeny of mice obtained from Dr. L. C. Strong in 1941.

Rates of Digestion, Gastric Emptying and Intestinal Absorption of Starch.

E. C. PAULSEN, P. F. FENTON,* AND H. B. PIERCE.

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Burlington, Vt.*

We have shown in previous studies^{1,2,3} that the rates of gastric emptying and intestinal absorption of carbohydrates were closely related. Our work has also indicated, contrary to the findings of Cori,⁴ that the rate of intestinal absorption of carbohydrates depended, among other things, upon the amount of administered carbohydrate. Much of our earlier work has been done with dextrose administered by forced feeding. This method has been called unphysiological by some workers, and it must be admitted that few animals habitually obtain their nourishment by stomach tube. On the other hand, we found it quite impossible to study dextrose absorption in short time intervals by using any method other than forced feeding. Starch, either in pure form, or as part of some food product, lends itself to studies with voluntary feeding. We have shown in an earlier study that rats can be made to consume limited amounts of such starch in a relatively short period of time.

The present study was undertaken to obtain further information on the fate of voluntarily ingested starch, to extend our previous experiments to other cereal products, and to determine the role salivary digestion plays in the utilization of starch by the rat.

Methods. This investigation was carried out with 3 preparations—(1) corn starch added to boiling water in amounts giving a concentration of 10%; (2) a breakfast cereal

from which the free sugar was removed by washing with water, followed by drying at 70°C (Cereal I); and (3) a breakfast cereal which contained no free sugar (Cereal II). The two breakfast cereals were fed as a paste, water being added in sufficient amounts to give a starch concentration of 10%. All 3 preparations were analyzed for starch, protein, fat, fiber, ash and moisture. As experimental animals we used white rats weighing between 140 and 200 g. These animals were fed the 3 starch preparations in amounts containing 247 or 317 mg of starch. In all other respects the procedure was the same as described by Fenton and Pierce.³

An effort was made to determine the extent of digestion in the alimentary tract above the pylorus. Some information was obtained by carrying out the standard osazone tests on the gastric contents. Since the presence of dextrans gave rise to abnormal crystal shapes, solubility in hot and cold water and in glacial acetic acid was used as the chief criterion. Additional information was obtained from a combination of Barfoed's and Benedict's qualitative tests.

Results and Discussion. The qualitative tests performed on the gastric and intestinal contents support the conclusion that the free sugar present in greatest amounts in the stomach was maltose while dextrose was found in the intestines. We have, therefore, calculated our results on this basis.

All pertinent data have been assembled in Table I. In general the rates of hydrolysis, emptying and absorption were closely related. The only exception to this was noted in the group receiving corn starch at the low level (247 mg of starch) with an absorption period of one hour. With this one exception the rates of hydrolysis, emptying and absorption of Cereal II and of corn starch were of the

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¹ Fenton, P. F., *Am. J. Physiol.*, 1945, **141**, 699.

² Birchall, E. F., Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1946, **145**, 610.

³ Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1947, **148**, 296.

⁴ Cori, C. F., *J. Biol. Chem.*, 1925, **66**, 691.

TABLE II.
Cross Adsorption Experiments.
Specific Adsorption of Mo Agglutinin and Failure of Mouse Erythrocytes to Adsorb Rabbit Erythrocyte Agglutinins from Human Sera.

Human serum type	Erythrocytes				
	Human			Mouse	
	A-Rh+	A-Rh—	B	Rabbit	AxCBAN (7) A (813)
A (50)	—	—	3+	2+	2+
A (46) before adsorption	—	—	3+	3+	2-3+
A (46) after adsorption with B cells	—	—	—	3+	2+
B (22) before adsorption	3+	—	—	3+	—
B (23) before adsorption	3+	3+	—	3+	3+
B (23) after adsorption with A-Rh+ cells	—	—	—	3+	3+
B (23) after adsorption with A-Rh— cells	—	—	—	3+	3+
A (46) adsorbed with mouse (AxCBAN 7) cells	—	—	3+	3+	—
B (23) adsorbed with mouse (C ₃ H 1534) cells	3+	3+	—	3+	—

The original discovery of the Rh factor⁸ was made by testing human beings with antiserum from rabbits which had been injected with Rhesus blood; in this fashion 85% of the individuals tested were positive. Gorer's² differentiation of the blood types in mice by reactions with human A serum was based on the quantitative or qualitative differences in the agglutinogens present in mouse erythrocytes. Since Gorer used himself as a source of type A serum, it is evident from his results that his blood contained Mo agglutinin. He apparently did not use a sufficiently large number of other individuals as sources of A serum to encounter one which did not contain Mo agglutinin. It is almost certain however, that the antibody in human A serum that he used, to study the differences in the agglutinin content of mouse erythrocytes, was Mo agglutinin. At least, the agglutinin in human sera that he used was absorbed by mouse erythrocytes and it was shown in this work that mouse red cells absorb

only Mo agglutinin and not α or β agglutinins or the rabbit cell agglutinin.

Summary and Conclusion. The performance of agglutination tests, using the blood cells of mice of 5 inbred strains and 8 types of F₁ and F₂ hybrid mice, have shown that human sera can be differentiated, apart from the usual intra-group reactions, by their ability to agglutinate mouse erythrocytes. The differentiation of human sera on this basis depended on the presence or absence of a specific agglutinin (Mo agglutinin). It was found to bear no specific relationship to either the A-B-O blood groups or the Rh factor. Five of the 37 human sera tested (approximately 14%) did not contain the Mo agglutinin. Mouse erythrocytes adsorbed the Mo agglutinin but not α and β agglutinins or the rabbit cell agglutinin. Human erythrocytes of the α and β group and rabbit red cells adsorbed their specific agglutinins, but not the Mo agglutinin.

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from which the free sugar was removed by washing with water, followed by drying at 70°C (Cereal I); and (3) a breakfast cereal which contained no free sugar (Cereal II). The two breakfast cereals were fed as a paste, water being added in sufficient amounts to give a starch concentration of 10%. All 3 preparations were analyzed for starch, protein, fat, fiber, ash and moisture. As experimental animals we used white rats weighing between 140 and 200 g. These animals were fed the 3 starch preparations in amounts containing 247 or 317 mg of starch. In all other respects the procedure was the same as described by Fenton and Pierce.³

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¹ Fenton, P. F., *Am. J. Physiol.*, 1945, **141**, 609.

² Birchall, E. F., Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1946, **146**, 610.

³ Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1947, **148**, 296.

⁴ Cori, C. F., *J. Biol. Chem.*, 1925, **60**, 691.

TABLE I.
Summary of Results Obtained with Rats Fed 3 Starch Preparations Fed at 2 Levels.*

Amount of starch fed	247 mg		317 mg	
Duration of experiment	1 hr mg	2 hr mg	1 hr mg	2 hr mg
Hydrolyzed				
Cereal I	137 \pm 7.3†	179 \pm 7.7	158 \pm 8.5	213 \pm 8.3
Cereal II	184 \pm 3.8	222 \pm 3.1	231 \pm 5.2	258 \pm 5.8
Corn starch	180 \pm 5.8	222 \pm 4.3	254 \pm 5.2	281 \pm 4.8
Emptied				
Cereal I	118 \pm 8.8	170 \pm 8.3	140 \pm 10.0	198 \pm 10.4
Cereal II	154 \pm 3.8	210 \pm 4.6	176 \pm 6.2	226 \pm 7.2
Corn starch	116 \pm 8.2	197 \pm 7.6	168 \pm 9.6	248 \pm 8.3
Absorbed				
Cereal I	117 \pm 8.8	170 \pm 8.3	130 \pm 9.4	195 \pm 10.1
Cereal II	148 \pm 4.4	210 \pm 4.6	158 \pm 6.2	220 \pm 7.8
Corn starch	115 \pm 8.0	197 \pm 7.6	165 \pm 9.8	248 \pm 8.3

* The data presented here were obtained on a total of 240 animals, each group containing from 15 to 30 animals. All values are expressed in terms of starch.

† Standard error

same order of magnitude. Cereal I was hydrolyzed, emptied and absorbed at a consistently slower rate.

The data presented here are in excellent agreement with our previous findings. It is again evident that increasing the size of the meal fed also increases the amount of starch hydrolyzed, emptied and absorbed. The fact that the rates of absorption decreased with time should not be emphasized too strongly since only relatively small amounts of carbohydrate remained unabsorbed during the second hour.

The intimate relationship of the rates of

hydrolysis, emptying and absorption and the effect of varying the size of the meal upon these rates support very well our earlier reports in connection with the utilization of dextrose. The results reported here were, however, obtained under conditions which permitted the elimination of forced feeding.

Conclusions. Three starch preparations were fed to a total of 240 animals. The rates of hydrolysis, emptying and absorption are closely related. Increasing the size of the test meal increases these rates. The data were obtained under conditions permitting the elimination of forced feeding.

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A Comparison of the Nutritive Value of Egg Proteins and Their Amino Acid Content.

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Since the early investigations of Osborne and Mendel¹ the high nutritive value of egg proteins has been recognized. Mitchell and Carman² found that whole egg protein fed at an 8% level in the diet had a biological value of 93 as compared with 74 for pork and 67 for wheat. Subsequently the same investi-

gators³ reported a biological value of 94 for the nitrogen of whole egg and 83 for the nitrogen of egg albumin. Sumner⁴ and others reported the biological value of whole egg protein as 94 when fed at a 5% level and 85 when fed at an 8% level and that the biological value was higher for young rats than

for older rats. It was also stated that egg proteins are superior to milk proteins in maintaining nitrogen balance of adult human subjects. Murlin and others⁵ gave 97 as the biological value of whole egg protein for human subjects as compared with values of 81, 84, and 83 for soy bean, beefsteak, and peanut proteins, respectively. Hoagland and Snider⁶ found that the average growth-promoting value of the proteins in dehydrated pork was 3.75 g gain in body weight per gram of protein consumed in tests with young rats as compared with a gain of 4.28 g for the protein in spray dried eggs. More recently Hoagland, Ellis, Hankins, and Snider⁷ stated that the superiority of egg to pork protein was due to the notably more cystine and methionine it contained. Supplementing pork protein with either cystine or methionine gave a growth promoting value equal to that of the protein in eggs.

Mitchell and Block⁸ have taken the amino acids of whole egg protein as a standard for comparison of other proteins in an effort to assay biological values on the basis of essential amino acid content. Egg albumin is deficient in both cystine and methionine in comparison with whole egg protein, no data on whole egg white protein or on whole yolk protein were given. Calvery and Titus⁹ have studied the sulfur, tryptophane, and cystine content of the whole white and whole yolk proteins and of egg albumin. The whole white protein was higher in all 3 substances than either the albumin or yolk proteins. The

whole white protein contained 1.66% sulfur and the yolk protein contained 1.19%. Munk and others¹⁰ found that the total egg protein was composed of 65% white protein and 35% yolk protein. On this basis the sulfur content of whole egg protein should be approximately 1.5%. Patton and Palmer¹¹ found the sulfur content of the whole egg protein to be 1.35%. Munk¹⁰ found practically the same cystine content in the white as in the yolk protein, 1.9% and 2.2% respectively. Methionine was determined by subtracting the cystine sulfur from the total sulfur and considering the difference as due to methionine. On this basis the whole white protein contained 6.6% and the yolk protein 3.0% methionine. The tryptophane content of the 2 proteins was identical, 1.4%, the phenylalanine content of the whole white protein was 6.2%, approximately 50% greater than the phenylalanine content of the yolk protein. Differences in the other essential amino acids were slight. No feeding studies were reported.

We have prepared whole egg, whole white, and yolk proteins and analyzed them for nitrogen, sulfur, cystine, cysteine, methionine, phenylalanine, tryptophane, threonine, histidine, lysine, and arginine. The same proteins were used in feeding experiments with young rats to determine their protein efficiency values. In addition 3 commercial egg proteins were run similarly.

Experimental. Preparation of proteins. The egg whites and yolks were separated and poured into acetone. After stirring vigorously they were allowed to stand overnight when the acetone was decanted and replaced with fresh acetone. This acetone treatment was repeated 4 times. The whole white protein was prepared similarly. After the final treatment with acetone all the proteins were fairly granular. Following the acetone treatment

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, **15**, 311; 1916, **20**, 35.

² Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1924, **60**, 613.

³ Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1926, **68**, 183.

⁴ Sumner, E. E., Pierce, H. B., and Murlin, J. R., *J. Nut.*, 1938, **10**, 37; Sumner, E. E., *J. Nut.*, 1938, **10**, 129; Sumner, E. E., and Murlin, J. R., *J. Nut.*, 1938, **10**, 141.

⁵ Murlin, J. R., Edwards, L. E., and Hawley, E. E., *J. Biol. Chem.*, 1944, **159**, 785.

⁶ Hoagland, R., and Snider, G. G., *Food Research*, 1946, **11**, 494.

⁷ Hoagland, R., Ellis, N. R., Hankins, O. G., and Snider, G. G., *J. Nut.*, 1947, **31**, 43.

⁸ Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, 1946, **163**, 599.

⁹ Calvery, H. O., and Titus, H. W., *J. Biol. Chem.*, 1934, **105**, 683.

¹⁰ Munks, B., Robinson, A., Beach, E. F., and Williams, H. H., *Poultry Sci.*, 1945, **24**, 549.

¹¹ Patton, A. R., and Palmer, L. S., *J. Nut.*, 1936, **11**, 129.

TABLE I.

Amino Acids in Egg Proteins.

All values, except nitrogen, are on the basis of the protein containing 16% nitrogen. Nitrogen values are expressed on the ash and moisture-free original protein.

Amino acid	Whole egg		Whole white	Whole yolk	Commercial whole egg		Commercial whole white
	I	II			I	II	
1. Cystine	%	%	%	%	%	%	%
2. Cysteine	2.26	2.25	2.48	1.82	1.33	2.11	2.33
3. Methionine	0.28	0.10	0.41	0.14	0.00	0.00	0.23
Phenylalanine	3.95	3.88	4.15	3.61	4.30	3.65	3.96
Tryptophane	4.46	4.50	5.42	3.73	4.84	4.87	5.29
Sulfur—total	1.29	1.42	1.40	1.16	1.47	1.35	1.56
Sulfur calculated from 1 + 2 + 3	1.57	1.56	1.78	1.30	1.38	1.40	1.62
Nitrogen	13.76	12.62	14.53	12.78	13.34	12.67	12.64
Arginine	6.54	6.46	5.55	6.98	6.40	6.59	6.29
Histidine	1.53	1.43	1.20	1.50	1.38	1.61	1.29
Lysine	5.14	5.05	4.83	5.81	5.09	5.13	5.20
Threonine	3.78	3.88	4.10	3.60	3.94	3.82	4.42

the proteins were treated overnight with ethyl alcohol. The alcohol was removed by filtration and the residues extracted with ethyl ether by allowing them to stand overnight in contact with the ether. The ether extraction was repeated twice. The final products were air dried.

The commercial proteins studied included a dried egg white and 2 dehydrated, defatted whole egg preparations, both made by the same manufacturer.

Analysis. Total nitrogen was determined on all samples by the macro Kjeldahl procedure. Total sulfur was determined by the method of Pollack and Partansky.¹² Phenylalanine, methionine, and tryptophane were determined, following alkaline hydrolysis, as described by Hess and Sullivan.¹³ Cystine and cysteine were determined by the method of Sullivan, Hess, and Howard¹⁴ as used by Hess and Sullivan.¹⁵ Threonine was determined by the method of Shinn and Nicolet.¹⁶

¹² Pollack, R. N., and Partansky, R. M., *Ind. and Eng. Chem., Anal. Ed.*, 1934, **6**, 330.

¹³ Hess, W. C., and Sullivan, M. X., *Ind. and Eng. Chem., Anal. Ed.*, 1945, **17**, 717.

¹⁴ Sullivan, M. X., Hess, W. C., and Howard, H. W., *J. Biol. Chem.*, 1942, **145**, 621.

¹⁵ Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, 1943, **151**, 625.

¹⁶ Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, 1941, **138**, 91.

Arginine, histidine, and lysine were determined by the methods described by Block and Bolling.¹⁷ The values given in Table I are expressed on the basis of the proteins containing 16% nitrogen since this method was used in determining the protein content of the diet. The nitrogen values in the table, however, are on the original sample corrected for moisture and ash.

Feeding experiments. Albino rats of the Sprague-Dawley strain were used. The diets were made up with the egg proteins to contribute 10% protein to the diet. The nitrogen of

TABLE II.
Composition of the Diet.

Constituent	%
U.S.P. No. 2 salt mixture	4
Wilson's 1:20 liver concentrate	1
Crisco	5
Corn oil	5
Protein—N \times 6.25 to contribute	10
Celcose and corn starch to make up 100%.	

Synthetic vitamins were added in the following quantities per 100 g of diet: alpha tocopherol 4.0 mg, 2-methyl-1,4-naphthoquinone 1.0 mg, thiamine hydrochloride 0.8 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.8 mg, niacin 4.0 mg, calcium pantothenate 4.4 mg, para-aminobenzoic acid 4.0 mg, choline chloride 200.0 mg, and inositol 21.6 mg. Vitamins A and D were fed separately, 3 drops of reference cod liver oil weekly.

In the diet containing 0.25% cystine an equal weight of corn starch was omitted.

¹⁷ Bosshardt, D. K., Ydse, L. C., Ayres, M. M., and Barnes, R. H., *J. Nut.*, 1946, **31**, 23.

TABLE III.
Growth of Weanling Rats on Egg Protein Diets.

Protein	No. of rats	Initial wt, g	Final wt, g	Gain, g	Food intake, g	PE ratio
Whole egg I	5	47.0*	97.0	50.0	151.4	3.30
		45.0	100.0	55.0	141.0	3.90
		55.0	108.0	53.0	177.0	2.99
Egg white	4	49.0	105.8	56.8	161.5	3.51
		54.0	120.0	66.0	177.0	3.73
		48.0	104.0	56.0	169.0	3.31
Yolk	4	46.8	96.0	48.8	151.0	3.23
		47.0	97.0	50.0	137.0	3.65
		49.0	106.0	57.0	191.0	2.98
Commercial egg white	8	49.8	104.1	54.4	161.1	3.37
		55.0	117.0	62.0	159.0	3.90
		45.0	87.0	42.0	168.0	2.50
Commercial whole egg I	9	49.4	90.9	41.4	148.4	2.80
		52.0	100.0	48.0	151.0	3.18
		44.0	83.0	39.0	159.0	2.45

* First value in each case is the average, followed by the values for the rats having the highest and lowest PE ratios in the group.

TABLE IV.
Growth of Weanling Rats on Egg Protein Diets.

Protein	No. of rats	Initial wt, g	Final wt, g	Gain, g	Food intake, g	PE ratio
Whole egg II	9	49.6*	82.1	32.6	104.4	3.12
		61.0	107.0	46.0	141.0	3.26
		60.0	94.0	34.0	124.0	2.74
Commercial whole egg II	9	48.8	73.2	24.6	100.9	2.33
		45.0	74.0	29.0	101.0	2.90
		50.0	68.0	18.0	103.0	1.75
Commercial whole egg II + 0.25% cystine	9	50.2	75.8	25.6	107.2	2.38
		44.0	70.0	26.0	89.0	2.92
		48.0	77.0	29.0	131.0	2.21

* First value in each case is the average, followed by the values for the rats having the highest and lowest PE ratios in the group.

the sample times 6.25 gave the protein content and the amounts of food consumed by the rats were carefully determined and recorded. Two series of feeding experiments were run. The average food intake and weight gains for a period of 2 weeks of each group of rats are given in Tables III and IV. Bosshardt and others¹⁸ found that the protein efficiency ratio* reached a maximum in approximately 10 days and state that this period is sufficiently long to permit reliable calculations. If the experiments are run for

a long period of time the PR ratio decreases.

Discussion. The first series of experiments, Table III, indicated that the whole white protein has a higher PE ratio than either the whole egg or yolk proteins. The commercial whole white protein has a PE ratio that is only slightly less than that of the laboratory whole white protein while commercial whole egg protein I has a markedly lower PE ratio than the laboratory prepared sample. In comparing the amino acid content of the laboratory prepared whole egg protein with that of commercial sample I it was noted that the sulfur amino acid content of the commercial sample was markedly lower. No

* The protein efficiency ratio (PE ratio) is calculated by dividing the weight gained by the grams of protein ingested.

marked differences were noted in the content of the other essential amino acids.

In an effort to determine whether the variation in total sulfur amino acid content, particularly cystine, would explain the difference in the PE ratio another series of feeding experiments were run. A new preparation of whole egg protein and another sample of commercial whole egg protein from the same producer were fed at the same level as previously. At the same time 0.25% cystine was added to the commercial whole egg protein diet and fed to another set of rats. These results are given in Table IV. The PE ratio of whole egg protein II was 3.13 as compared with the 3.3 in the first series while commercial whole egg protein II was 2.33 compared with 2.8 in the first series. The commercial whole egg protein II plus cystine had a PE ratio of 2.38, practically identical with that of the diet without the added cystine. It will be noted from Table I that whole egg protein II has an amino acid content the same as that of Sample I within the limits of experimental error. Commercial whole egg protein II contains slightly more cystine than commercial Sample I and slightly less methionine, there is little variation in all the samples of whole egg proteins in the other essential amino acids tested. It is apparent that the addition of cystine has not improved the PE ratio of the commercial whole egg protein. The only essential amino acids which were not determined in the proteins are leucine, isoleucine, and valine. These, according to Mitchell and Block,⁸ are present in such large amounts in whole egg protein that it is difficult to imagine any lack of these amino acids in any of the samples.

These results emphasize the difficulty of assaying the nutritional values of proteins by analyzing for the essential amino acids. In spite of the similarity of the amino acid content of the commercial whole egg and laboratory whole egg proteins, except for cystine, they are biologically different, even when the commercial sample is reenforced with cystine.

Summary. The proteins of whole egg, whole egg white, and yolk, were prepared and analyzed for cystine, methionine, phenylalanine, tryptophane, threonine, arginine, histidine, lysine, sulfur, and nitrogen. These proteins were incorporated into diets at a 10% level and fed to young rats. The protein efficiency ratio (gain in weight per gram of protein ingested) was determined after a 2-week feeding period. Commercial preparations of whole egg and whole white protein were similarly analyzed and their PE ratio determined. The PE ratio of the whole white protein was higher than that of either the whole egg or the yolk proteins. The PE ratio of the commercial whole white protein was almost as high as that of the laboratory prepared sample. The PE ratio of commercial whole egg protein was markedly lower than that of the laboratory prepared sample. The amino acid content of all the whole egg proteins were quite similar with the exception of the cystine content of the commercial samples which was lower than that of the laboratory samples. When the commercial whole egg protein II was reenforced with 0.25% cystine the PE ratio was not improved. Other factors in addition to the amino acid content apparently play a role in the biological value of proteins.

16373 P

Effect of Potassium on the Ventricular Deflections of the Electrocardiogram in Hypertensive Cardiovascular Disease.*

J. MARION BRYANT. (Introduced by Frank N. Wilson.)

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Many reports have been published concerning the effects of potassium on the electrocardiogram in various clinical states¹⁻¹³ and in experimental animals.¹⁴⁻¹⁷ These have been concerned chiefly with its effect upon the T-wave. Relatively little attention has

been devoted to its effect upon the QRS complex except in those cases in which toxic doses were given. Inspection of the published records, however, shows that in many instances a reduction in the size of the QRS deflections followed the administration of this drug. We refer particularly to instances in which potassium was given to patients with hypertensive heart disease. In the articles referred to the QRS changes were not mentioned in the texts or legends. In fact in some of these it was reported that no change in the form of these deflections took place.^{4,13} In a previously reported study¹⁸ sodium restriction in hypertensive vascular disease was followed by retrogressive electrocardiographic changes unaccompanied by a significant change in blood pressure. This raised the question as to whether a relative increase in tissue potassium secondary to sodium restriction was responsible for these electrocardiographic phenomena. Consequently the effect of potassium alone upon the electrocardiogram in cases of hypertension was investigated. Potassium in the form of the chloride, dibasic phosphate, or citrate was administered, on 25 occasions, to patients with abnormal electrocardiograms characteristic of hypertensive vascular disease, and, on ten occasions, to normal subjects. Electrocardiograms were obtained immediately before and at times ranging from 15 minutes to 3 hours after the ingestion of 5 to 20 g of one of the potassium salts mentioned. In several instances 10 to 24 g of potassium salts daily had been prescribed previously and the patient had continued on this dosage up to the time of the test.

In every instance in which potassium was

* The observations upon which this paper is based were carried out with the aid of grants to Frank N. Wilson from the Horace H. Rackham School of Graduate Studies and the S. S. Kresge Foundation.

¹ Bamber, J. M., *Am. J. Med. Sci.*, 1935, **189**, 681.

² DeWesselow, O. L. V. S., and Thomson, W. A. R., *Quart. J. Med.*, 1939, **8**, 361.

³ Thomson, W. A. R., *Lancet*, 1939, **1**, 808.

⁴ Thomson, W. A. R., *Brit. Heart J.*, 1939, **1**, 269.

⁵ Stewart, H. J., Smith, J. J., and Milhorat, A. I., *Am. J. Med. Sci.*, 1940, **199**, 789.

⁶ Stewart, H. J., and Smith, J. J., *Am. J. Med. Sci.*, 1941, **201**, 177.

⁷ Keith, N. M., Osterberg, A. E., and Burchell, H. B., *Ann. Int. Med.*, 1942, **16**, 879.

⁸ Keith, N. M., and Osterberg, A. E., *Proc. Staff Meet., Mayo Clinic*, 1946, **21**, 385.

⁹ Sampson, J. J., Albertson, E. C., and Kondo, B., *Am. Heart J.*, 1943, **26**, 164.

¹⁰ Sharpey-Schafer, E. P., *Brit. Heart J.*, 1943, **5**, 80.

¹¹ Sharpey-Schafer, E. P., *Brit. Heart J.*, 1943, **5**, 85.

¹² Keith, N. M., Burchell, H. B., and Baggenstoss, A. H., *Am. Heart J.*, 1944, **27**, 817.

¹³ del Castillo, E. B., and Onativia, E. A. y A., *Medicina*, 1945, **6**, 471.

¹⁴ McLean, F. C., Bay, E. B., and Hastings, A. B., *Am. J. Physiol.*, 1933, **103**, 72.

¹⁵ Winkler, A. W., Hoff, H. E., and Smith, P. K., *Am. J. Physiol.*, 1938, **124**, 478.

¹⁶ Winkler, A. W., Hoff, H. E., and Smith, P. K., *Am. J. Physiol.*, 1939, **127**, 430.

¹⁷ Chamberlain, F. L., Scudder, J., and Zwemer, R. L., *Am. Heart J.*, 1939, **18**, 453.

¹⁸ Bryant, J. M., and Blecha, E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 227.

given to a patient with hypertensive heart disease accompanied by the characteristic changes in the electrocardiogram there was a reduction in voltage of the QRS deflections which persisted for several hours. Frequently there was also a reduction in the length of the QRS interval. In the vast majority of cases the T-wave became less inverted or upright when it was originally inverted, or taller when it was originally upright. Serial electrocardiographic observations showed that following the administration of potassium the electrocardiogram passes in reverse order through the same series of configurations that it passes through in the course of the development of the changes characteristic of hypertensive heart disease. In a few instances the T-wave became more deeply inverted after potassium even though the voltage of the QRS deflections was reduced. In the majority of instances the mean axis of the QRS shifted to the right of its original position while that of the T-wave was shifted to the left. The magnitude of the ventricular gradient was usually increased and its direction was shifted slightly to the right.

In normal subjects the administration of potassium was followed by electrocardiographic changes similar in character but less pronounced than those seen in cases of hypertension. In one patient with left ventricular enlargement due to luetic aortic insufficiency,

but with no definite increase in blood pressure, the administration of potassium was followed by pronounced toxic symptoms and transient left bundle branch block.

In hypertensive vascular disease sodium restriction alone is followed by electrocardiographic changes identical with those produced by the administration of potassium. No close correlation between the changes in blood pressure and the changes in the electrocardiogram is apparent. It should be pointed out that the electrocardiographic changes following sympathectomy in hypertensive patients are of the same type, and, that in this case also these changes are not always very closely related to the effects on the blood pressure.

In calling attention to the similarity between the changes in the electrocardiogram produced by the administration of potassium in cases of hypertensive heart disease, and the changes in the electrocardiogram which often follow sympathectomy or the restriction of sodium in cases of the same kind, we do not imply that the mechanism by which these electrocardiographic changes is brought about is necessarily the same in both instances. This is a question which can be decided only by future investigations.

The potassium dibasic phosphate used in this study was supplied through the generosity of Parke, Davis and Company.

16374 P

A Gaseous Nitrogen Elimination Test to Determine Pulmonary Efficiency.

WALTER M. BOOTHBY, GUNNAR LUNDIN,* AND H. FREDERIC HELMHOLZ, JR.

From the High Altitude Laboratory, Mayo Aero Medical Unit, Mayo Clinic and Mayo Foundation, Rochester, Minn.

Nearly all physiologists interested in respiration have contributed to our knowledge of nitrogen elimination produced by decompression or by oxygen inhalation. As space prevents an adequate review of the literature,

we can only refer the reader to typical references of studies by Haldane and Priestley,¹

¹ Haldane, J. S., and Priestley, J. G., *Respiration*, New Haven, Yale University Press, 1935, 493 pp.

² Berggren, S. M., *Acta physiol. Scandinav.*, 1942, 4 (Suppl. 11).

* Department of Aviation Physiology, University of Lund, Lund, Sweden.

Berggren,² Behnke,³ Bornstein,⁴ Lundsgaard and Van Slyke,⁵ Christie,⁶ Courmand and his associates,⁷ and Bateman.⁸

As a result of the ease and rapidity of analysis possible with the nitrogen meter devised by Lilly and Anderson⁹ of the Johnson Foundation it is now possible to make, as frequently as desired, readings of the nitrogen content of oxygen-nitrogen mixtures. For the principle and details of this nitrogen analyzer the reader should consult the original paper. For our use Baldes enlarged the lower part of the scale (0 to 15%) so that differences in the nitrogen content of CO₂-free nitrogen-oxygen mixtures at constant temperature and saturated with water vapor can be determined with an accuracy of 0.05% points provided duplicate analyses in Haldane high oxygen gas-analyzers are made at the beginning and end of each experiment for the purpose of calibration; the intermediate readings are linear.

The respiratory apparatus used is a closed circuit system consisting of a spirometer, soda-lime container, filter and a blower with corrugated tube connections to the valve attached to mask or mouthpiece.

The spirometer records both respiratory movements and ventilation rate and has a fan on the floor for rapid mixing of gases. For an experiment it is filled with 40 to 60 liters of oxygen (99.6 \pm 0.1% purity) so that at the end of the 30-minute experiment the nitrogen content does not exceed 8 to 10%. Wet and dry bulb thermometers are placed in the air stream just as the circulating mix-

ture leaves the gasometer. A sampling tube leads to the nitrogen meter which draws out approximately 6 cc per minute. The blower produces a flow of 80 to 100 liters per minute through the closed circuit system. Therefore the over-all time lag is less than 0.2 minute from the moment the expired air enters the system to the meter reading of the nitrogen content of the mixed gases.

The experiment itself is conducted like a basal metabolism test except that it lasts 30 minutes. The valve to start the experiment must be turned exactly at the bottom of the subject's natural expiration to avoid a bothersome correction. Just before the experiment is ended the vital capacity is determined by 2 cycles of maximal inspiration and expiration; the spirometer tracing is then continued for 5 minutes to establish accurately the average expiratory level from which the different pulmonary subdivisions are calculated.

The accumulated nitrogen elimination (BTPS) is a simple calculation and should be made for 15 to 20 different points in the 30 minute experimental period. For plotting we have found the use of 2 by 3 cycle log-log paper very convenient, for the following reasons: 1. The time scale covers 3 log units (0.2 to 30 minutes). 2. As first shown by the experiments of Boothby, Lovelace and Benson,^{10,11} the body tissue nitrogen, both when the subject is at rest and when he is at work, when accumulated and plotted on log-log paper forms straight lines for more than 200 minutes; the slope of the line is in the main dependent on the rate at which the venous blood returns to the lungs. 3. The accumulated nitrogen coming from the air in the lungs rises very rapidly from the beginning of the experiment; when the pulmonary nitrogen has been completely eliminated, a sharp angle or knee is made as the further

³ Behnke, A. R., *U. S. Naval Med. Bull.*, 1937, **35**, 219.

⁴ Bornstein, A., *Z. f. exp. Path. u. Therap.*, 1918, **20**, 495.

⁵ Lundsgaard, Christen, and Van Slyke, D. D., *J. Exp. Med.*, 1918, **27**, 63.

⁶ Christie, R. V., *J. Clin. Invest.*, 1932, **11**, 1099.
⁷ Courmand, Andre, Baldwin, Eleanor deF., Darling, R. C., and Richards, D. W., Jr., *J. Clin. Invest.*, 1941, **20**, 681.

⁸ Bateman, J. B., *Proc. Staff Meet., Mayo Clin.*, 1946, **21**, 112.

⁹ Lilly, J. C., and Anderson, T. F., National Research Council, Division of Medical Science, CAM Report No. 299, 1944.

¹⁰ Boothby, W. M., Lovelace, W. R., II, and Benson, O. O., *J. Aeronautical Sci.*, 1940, **7**, 524.

¹¹ *Physiology of Flight; Human Factors in the Operation of Military Aircraft*. A compendium of lectures and demonstrations given to Army Air Force personnel. The Aero Medical Research Laboratory, Experimental Engineering Section, Materiel Center, Wright Field, Dayton, Ohio, 1940-1942, p. 27, Fig. 15.

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⁹ Lilly, J. C., and Anderson, T. F., National Research Council, Division of Medical Science, CAM Report No. 299, 1944.

¹⁰ Boothby, W. M., Lovelace, W. R., II, and Benson, O. O., *J. Aeronautical Sci.*, 1940, **7**, 524.

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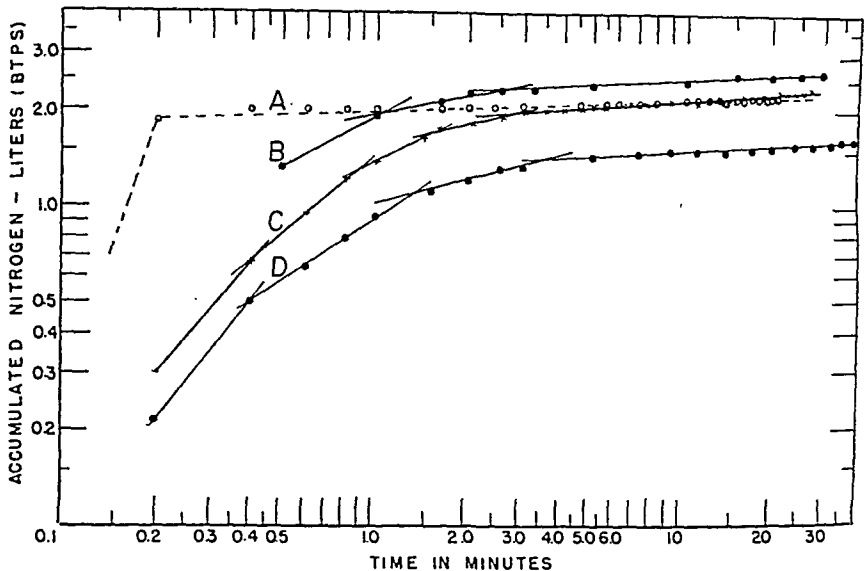


FIG. 1.

Characteristic curves plotted on log-log paper. Subject: man, aged 37 years; height 183 cm; weight 83.6 kg; surface area 2.07 square meters.

- A. Subject started with 4 maximal respirations, then breathed normally.
 - B. The largest value for functional residual air volume in the series. Normal breathing.
 - C. The usual value for functional residual air volume in the series. Normal breathing.
 - D. The smallest value for functional residual air volume in the series. Normal breathing.
- In B, C, and D the nitrogen washout time was between $2\frac{1}{2}$ and $3\frac{1}{2}$ minutes.
In A the washout time with 4 deep breaths was 0.2 minute.

accumulation of tissue nitrogen begins to form the straight line that continues for the duration of the experiment. The first part of the curve, representing essentially pulmonary nitrogen, may be in 2 or sometimes 3 phases which are important indicators of the degree and cause of respiratory inefficiency in some pulmonary diseases. 4. The final and characteristic bend or knee in the nitrogen elimination curve on log-log paper serves therefore as a convenient method of separating pulmonary from tissue nitrogen (Fig. 1). This graphic method permits one also to estimate, when desired, the comparatively small amount of tissue nitrogen that is eliminated along with the pulmonary nitrogen.

To calculate the functional residual air volume (BTPS) first read from the log-log plot the volume of nitrogen corresponding to the final bend or knee; second, divide this volume by the usual fraction of nitrogen normally present in alveolar air, 0.80, less the fraction of nitrogen in the inspired air at the time this knee is formed (which is

usually about 0.05). The true residual air volume is obtained by subtracting the supplementary (reserve) air volume; the complementary and supplementary air volumes, the sum of which is the vital capacity, are obtained from the spirometer respiratory curve; the mean expiratory level is the base line from which the measurements are made. The total capacity is the vital capacity plus the true residual air volume. All air volumes are expressed at body temperature and pressure, saturated with water vapor (BTPS). They therefore represent the true size of the lung space available for respiration.

The oxygen consumption is determined as in any closed circuit system (after proper corrections) from the decrease in the spirometer volume during the experimental period.

The ventilation rate is also read from the spirometer tracing and the ratio between oxygen consumption and ventilation rate is calculated.

The efficiency of the subject's natural method of breathing is determined by the

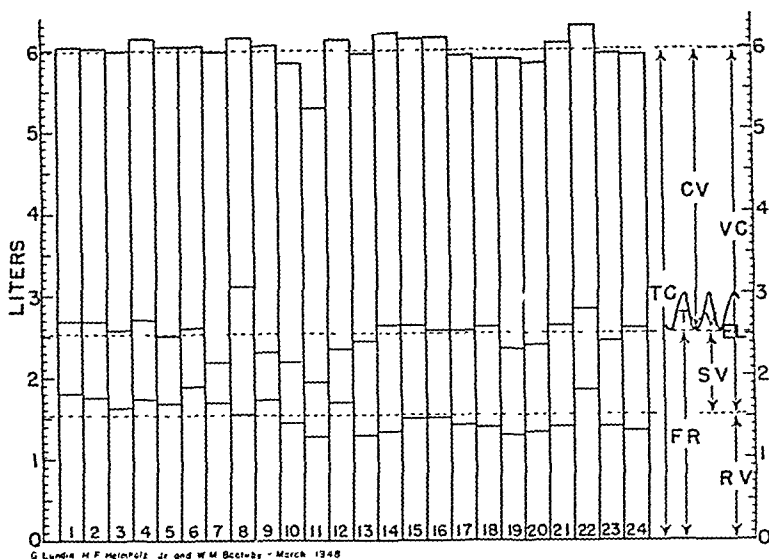


FIG. 2.

Variations in the subdivisions of lung capacity as found in a series of 24 experiments on a normal subject at rest in a sitting posture. Subject: man, aged 37 years; height 183 cm; weight 83.6 kg; surface area 2.07 square meters. TC—total capacity; FR—functional residual air volume; CV—complementary air volume; SV—supplementary air volume; VC—vital capacity; RV—true residual air volume.

washout time required for elimination of the nitrogen that was in the pulmonary air; that is, the time after the subject begins breathing oxygen to the point on the plot where the knee or final bend occurs. In normal subjects under basal conditions this varies between 2 and 3.5 minutes; ventilatory inefficiency is increasingly severe as the washout time increases beyond 4 minutes; some patients with marked emphysema require 10 to 12 minutes to clear their lungs of pulmonary air nitrogen.

In a normal subject under the same conditions the results are reproducible, as seen in Fig. 2.

Summary. An improved method for determining the nitrogen washout time with measurement of the various subdivisions of the pulmonary volume is described. The method is proving helpful in the differential diagnosis of pulmonary disease and in estimating the functional changes produced by intrathoracic operations.

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